Geographic variation in nrDNA and four cpDNA regions of *Juniperus excelsa*: Analysis of new records from Bulgaria, Cyprus and southwestern Turkey

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ABSTRACT

Sequencing of nrDNA, plus four cp DNA regions: petN-psbM, trnS-trnG, trnD-trnT and trnL-trnF of newly acquired samples of *J. excelsa* from Bulgaria, Cyprus and Turkey showed little variation in *J. excelsa* (sensu stricto), except for the unusual situation in Lebanon, where *J. excelsa* and *J. polycarpos* (and likely *J. p.* var. *turcomanica*) grow near each other and may be hybridizing. The genetic composition of the eastern-most populations of *J. excelsa* in Turkey is unknown and deserves further study. Published on-line **www.phytologia.org** *Phytologia 98(1): 1-7 (Jan. 5, 2016).* ISSN 030319430

KEY WORDS: *Juniperus excelsa, J. polycarpos* var. *polycarpos, J. polycarpos* var. *turcomanica, J. seravschanica*, DNA sequencing, nrDNA, petN-psbM, trnS-trnG, trnD-trnT, trnL-trnF.

Recently, Adams et al. (2014a) examined *J. excelsa* and putative *J. polycarpos* from the eastern Mediterreanea eastward into Azerbaijan. They reported that putative *J. excelsa* from Azerbaijan is *J. polycarpos* (= *J. excelsa* subsp. *polycarpos*). Two Lebanon *Juniperus* populations from Afqa (1306 m) and Wadi El Njass (2287 m), previously shown to be divergent in their microsatellites, were shown to be *J. excelsa* and *J. polycarpos,* respectively. This was the first report of the occurrence of *J. polycarpos* in Lebanon.

Analyses of the volatile leaf oils of *J. excelsa* (Adams et al. 2014b) revealed that the oils from Bulgaria and Greece were higher in α-pinene, limonene and β-phellandrene than populations from Turkey, Cyprus and Lebanon. Otherwise, there was little variation in the oils between these populations. Cedrol was a major component in each of the populations, ranging from 22.6 to 29.3%. Analysei of *J. polycarpos* var. *polycarpos* from Azerbaijan revealed the presence of high cedrol and zero cedrol chemotypes. The high cedrol chemotype was similar to the oil from Armenia. The Azerbaijan zero cedrol chemotype was similar to the oil from El Njass, Lebanon.

The aforementioned reports were preceded by the Douaihy et al. (2011) study in which 3 microsatellites of putative *J. excelsa*, reported that the Nei genetic distance separated their 12 populations into 3 groups: Lebanon (Leb 1, 2, 4, 5), Lebanon (Leb 3, 6) and the Crimea, Cyprus, Greece and Turkey populations. PCO of the data removed 38.8% and 27.5% on the first two axes. Ordination clearly showed: Lebanon (Leb 1, 2, 4, 5), Lebanon (Leb 3, 6) and the Crimea, Cyprus, Greece and Turkey populations. El Njass (Leb 3, 2287 m) and Aarsal (Leb 6, 2180 m) are from higher elevations in Lebanon.

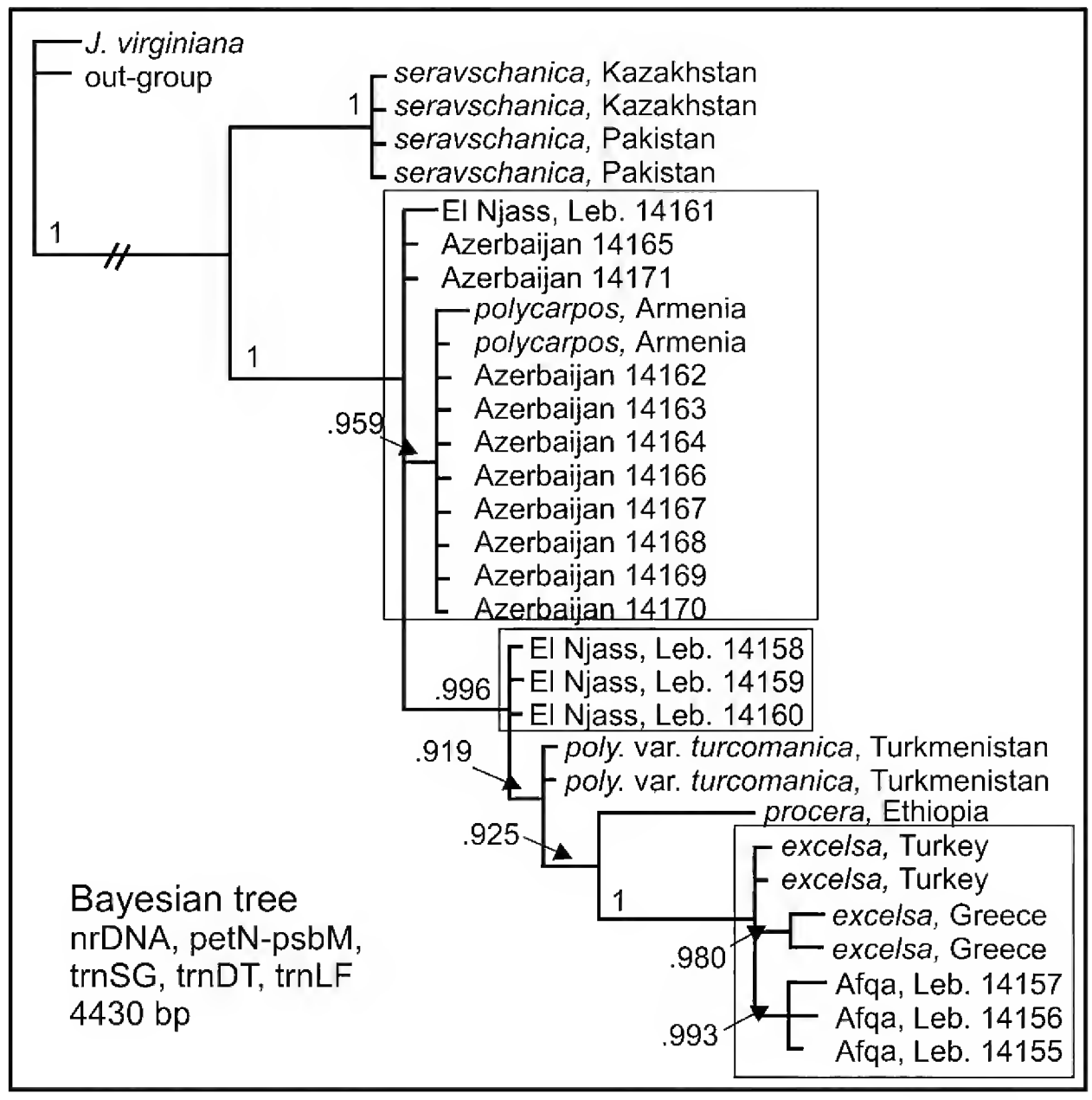

Figure 1. Bayesian tree based on nrDNA (ITS) and four cp regions: petN-psbM, trnS-trnG, trnD-trnT and trnL-trnF (4430 bp). Numbers at the branch points are posterior probabilities.

Examination of plants (RPA) from Afqa, 1300 m and El Njass, 2287 m, found that Afqa plants had very fine, small leaves. The Afqa leaves were bluish green similar to *J. excelsa* from Greece. The leaves of the El Njass plants were larger, coarse and yellowish green similar to *J. polycarpos* from Armenia and *J. p.* var. *turcomanica* from Turkmenistan.

In a comprehensive study of the reproductive ecology of *Juniperus* in Lebanon, Douaihy et al. (2013) reported differences between the higher (El Njass, Aarsal) and lower (Afqa, etc.) populations in cones density classes, frequencies of adult and juvenile trees, and dioecious (El Njass, Aarsal) vs. monecious (Afqa, etc.) individuals. Interestingly, Adams (2014) describes *J. excelsa* as monoecious or dioecious and *J. polycarpos* as dioecious.

Juniperus excelsa M.-Bieb. grows from Greece (Fig. 2). Farjon (2005, 2010) treated *J. polycarpos, J. p.* var. *seravschanica* and *J. p.* var. *turcomanica* as *J. excelsa* subsp. *polycarpos*. However, Adams et al. (2008), Adams and Schwarzbach (2012) and Adams (2013), utilizing DNA sequence data, recognized *J. excelsa,* in addition to *J. polycarpos, J. p.* var. *turcomanica* and *J. seravschanica.* Adams

and Hojjati (2012) and Adams, Hojjati and Schwarzbach (2014), using sequences from 4 gene regions, did not find *J. excelsa* in Iran, but did confirm *J. polycarpos, J. p.* var. *turcomanica* and *J. seravschanica* in Iran. Putative *J. excelsa* from Qushchi, in extreme northwest Iran, had none or only one SNP difference compared with *J. polycarpos* var. *polycarpos* from Armenia and was concluded to be *J. polycarpos* (Adams and Hojjati, 2012). Adams et al. (2014a) found that putative *J. excelsa* in Azerbaijan was, in fact, *J. polycarpos* or in one case, a putative hybrid.

The distribution of *J. excelsa* in Bulgaria, Cyprus and southwestern Turkey has proved difficult to determine by modern methods of DNA sequencing and leaf essential oil data, due to the lack of samples from these regions. Recently, materials were obtained of *J. excelsa* from Bulgaria, Cyprus and southwestern Turkey. This afforded the opportunity to further examine geographic variation in the DNA sequences of *J. excelsa*.

The purpose of the paper is to examine nrDNA, and 4 cp DNA regions: petN-psbM, trnS-trnG, trnD-trnT and trnL-trnF and report on variation in *J. excelsa*.

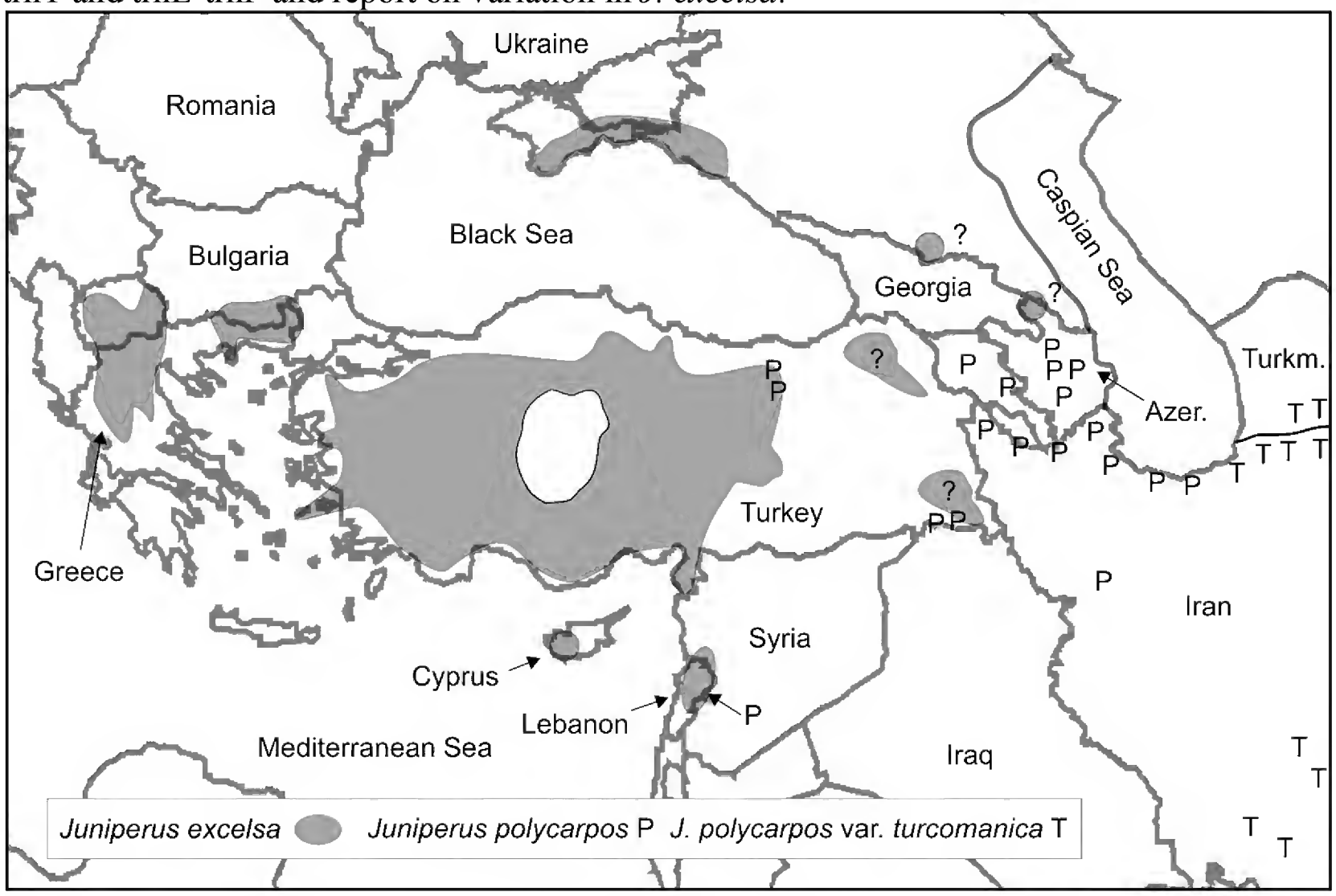

Figure 2. Distribution of *J. excelsa*, *J. polycarpos* var. *polycarpos* (P) and *J. p.* var. *turcomanica* (T) . Questionable locations of *J. excelsa* and *J. polycarpos* are indicated by E? and P? (modified from Adams, et al., 2014a).

MATERIALS AND METHODS

Plant material -

J. excelsa:

Bulgaria, Central Rhodopes, above the town of Kritchim, Reserve "Izgorialoto Gune", 42° 01' 22.0" N; 24° 28' 03.1" E, 356 m, *Alex Tashev, 2012-1-JE -5-JE*, 1 Sep 2012, Lab Acc. *Adams 13720-13724,*; **Cyprus**: 34° 57' 45.82" N, 33° 59' 55.33" E, elev. 1461m, *Salih Gucel ns*, 3 July 2015, Lab Acc. *Adams 14570-14574*; **Greece**: Lemos, ca 40° 49' N, 21° 03' E, 1100m, *Adams 5983-5985, 5987*; **Lebanon**: Afqa, 34° 04' 58.12"N, 35° 53' 08.52" E, 1306 m, *Bouchra Douaihy 1-3*, 4 Nov 2013, Lab Acc. *Adams 14155-14157*; **Turkey**: Antalya-Manavgat, Köprülü Canyon National Park, 37° 20' N; 31° 16' E, elev. 550 m,

Tuğrul Mataraci 2015-18, 24 May 2015, Lab Acc *Adams 14569*; Isparta-Eğirdir, junction of Kasımlar-Sütçüler road, 37° 28' N; 30° 59' E, elev. 1180 m, *Tugrul Mataraci, 2015-7*, 24 May 2015, Lab Acc. *Adams 14596*; ~40 km north of Eskişehir, with Oaks, 39° 58.307'N; 30° 41.045' E, Turkey, 820m, *Adams 9433-9435;*

J. polycarpos:
Armenia: Lake Sevan, 1900m, *Adams 8761-8763*; **Azerbaijan**, 40° 44' 41.05" N; 47° 35' 19.14" E, 177-231m, *Vahid Farzaliyev 1-10*, Dec 2013, Lab Acc. *Adams 14162-14171*; **Lebanon:**, Wadi El Njass, 34° 20' 47.79"N, 36° 05' 45.54"E, 2287m, *Bouchra Douaihy 4-7*, 14 Nov 2013, Lab Acc. *Adams 14158-14161*;

J. polycarpos var. *turcomanica*: **Turkmenistan**: Kopet Mtns., 38° 25.12'N, 56° 58.80'E, 1535 m, 22 May 1999, *Adams 8758-8790*;

J. procera: **Ethiopia**: on the road to Guder, ca. 40 km w of Addis Abba, ca. 9° 02'N, 38° 24' W, 2400 m, *Adams 6184-6188*;

J. seravschanica: **Pakistan**: near Quetta, Baluchistan, *A. Hafeez Buzdar ns*, 6 Apr 1998, Lab Acc. *Adams 8483-8485*; **Kazakhstan**: west end of Talasskiy Ala-Tau Range, ca. 2 km S. of Dzhabagly, 42^0 24.53'N, 70^0 28.50'E, 1770m, 12 Sept 1997, *Adams 8224-8226*.
Voucher specimens deposited in the Herbarium, Baylor University (BAYLU).

One gram (fresh weight) of the foliage was placed in 20 g of activated silica gel and transported to the lab, thence stored at -20^o C until the DNA was extracted. DNA was extracted from juniper leaves by use of a Qiagen mini-plant kit (Qiagen, Valencia, CA) as per manufacturer's instructions.

Amplifications were performed in 30 µl reactions using 6 ng of genomic DNA, 1.5 units Epi-Centre Fail-Safe Taq polymerase, 15 µl 2x buffer E (petN, trnD-T, trnL-F, trnS-G) or K (nrDNA) (final concentration: 50 mM KCl, 50 mM Tris-HCl (pH 8.3), 200 µM each dNTP, plus Epi-Centre proprietary enhancers with 1.5 - 3.5 mM $MgCl_2$ according to the buffer used) 1.8 µM each primer. See Adams, Bartel and Price (2009) for the ITS and petN-psbM primers utilized. The primers for trnD-trnT, trnL-trnF and trnS-trnG regions have been previously reported (Adams and Kauffmann, 2010).

The PCR reaction was subjected to purification by agarose gel electrophoresis. In each case, the band was excised and purified using a Qiagen QIAquick gel extraction kit (Qiagen, Valencia, CA). The gel purified DNA band with the appropriate sequencing primer was sent to McLab Inc. (San Francisco) for sequencing. Sequences for both strands were edited and a consensus sequence was produced using Chromas, version 2.31 (Technelysium Pty Ltd.) or Sequencher v. 5 (genecodes.com). Sequence datasets were analyzed using Geneious v. R7 (Biomatters. Available from http://www.geneious.com/), the MAFFT alignment program. Further analyses utilized the Bayesian analysis software Mr. Bayes v.3.1 (Ronquist and Huelsenbeck 2003). For phylogenetic analyses, appropriate nucleotide substitution models were selected using Modeltest v3.7 (Posada and Crandall 1998) and Akaike's information criterion. Minimum spanning networks were constructed from mutational events (ME) data using PCODNA software (Adams et al., 2009; Adams, 1975; Veldman, 1967).

RESULTS AND DISCUSSION

Sequencing nrDNA (ITS) and four cp regions petN-psbM, trnS-trnG, trnD-trnT and trnL-trnF yielded 4430 bp of data. The Bayesian consensus tree shows *Juniperus seravschanica, J. polycarpos, J. p.* var. *turcomanica, J. procera* and *J. excelsa* in well-supported clades. *J. excelsa* samples, newly

collected from Bulgaria, Cyprus, and sw Turkey, are in a clade with other *J. excelsa* (Fig. 3). There is some minor variation among the *J. excelsa* samples, mostly notably in the Afqa, Lebanon population.

All of the *J. polycarpos* samples from Azerbaijan are closely related with *J. polycarpos*, Armenia along with the El Njass, Lebanon (Adams 14161) sample (Fig. 3). Three other El Njass samples (*Adams 14158, 14158, 14160*) appear to be intermediate between *J. polycarpos and J. p.* var. *turcomanica* (Fig. 3).

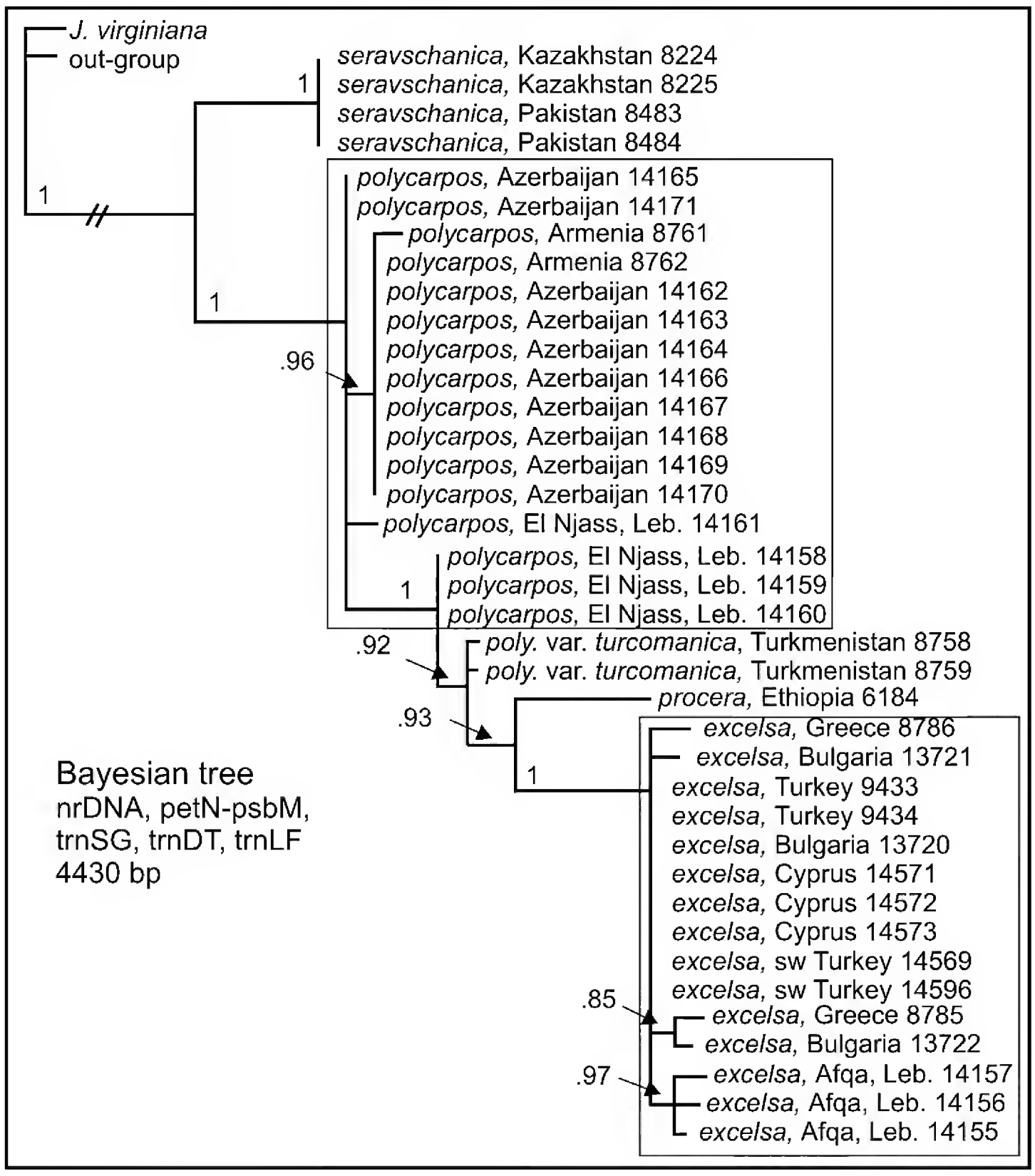

Figure 3. Bayesian analysis based on nrDNA, petN-psbM, trnSG, trnDT and trnLF. Numbers at the branch points are posterior probabilities.

Overlaying a minimum spanning network onto a distribution map gives one a perspective of the geographic trends (Fig. 4). The newly sampled *J. excelsa* populations from Bulgaria, Cyprus and sw Turkey are identical or nearly identical to *J. excelsa* of Eskisehir, Turkey (Fig. 4). Both the Cyprus and southwestern Turkey populations of *J. excelsa* showed no differences (Fig. 4). The Bulgaria *J. excelsa* differed by none or one difference from Eskisehir, Turkey (Fig. 4).

As previously reported (Adams et al., 2014a), the Afqa, Lebanon *J. excelsa* population differs by 2 MEs from Eskisehir, Turkey, which in turn, differs by only 1 ME from the Lemos, Greece population (Fig. 4). The other Lebanon populations that group with Afqa are probably *J. excelsa*.

However, the Wadi El Njass, Lebanon (2287 m) population, although near Afqa, is *J. polycarpos* and differs by 1 to 3 MEs from *J. p.* var. *turcomanica*, Turkmenistan and by 1 to 2 MEs from *J. polycarpos,* Armenia (Fig. 4). The *J. excelsa*, Afqa population is only about 100 - 150 km from other *J. excelsa* populations (Fig. 4), but the Wadi El Njass, *J. polycarpos* population is 700 to 1000 km from the nearest *J. polycarpos* population, still, it differs by only 1 to 3 MEs.

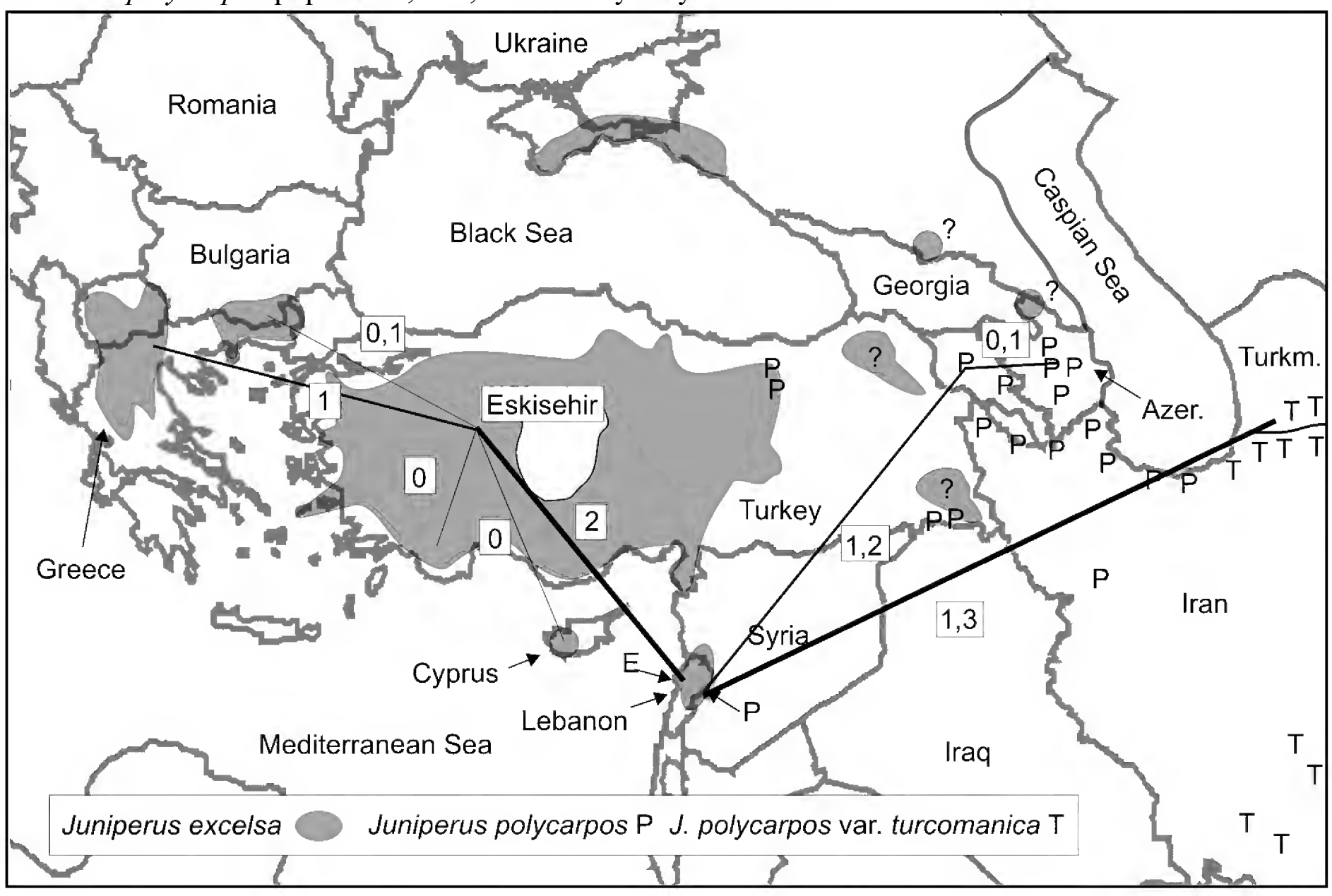

Figure 4. Minimum spanning network mapped onto the distributions of *J. excelsa and J. polycarpos*. Numbers next to lines are the number of MEs (Mutational Events = base substitutions plus indels).

Clearly, DNA sequencing shows that *J. excelsa*, as sampled in this study, is a fairly uniform species, except for the unusual situation in Lebanon, where *J. excelsa* and *J. polycarpos* (and likely *J. p.* var. *turcomanica*) grow near each other and may be hybridizing. The genetic composition of the eastern-most populations of *J. excelsa* in Turkey is unknown and deserves study.

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First molecular evidence that *Juniperus communis* var. *communis* from the eastern hemisphere is growing in the northeastern United States:

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ABSTRACT

Scattered in floras of New England and West Virginia are reports of upright trees of *Juniperus communis*. The diagnostic DNA sequence, petN-psbM, distinguishes all eastern hemisphere *J. communis* varieties from those in the western hemisphere. Analyses of petN-psbM for several upright J. *communis* trees from New England and West Virginia confirmed that trees from Maine and West Virginia were *J. communis* var. *communis* and were likely seed from trees cultivated in cemeteries and gardens. However, four trees from Massachusetts and one from Vermont proved to be upright forms of *J. communis* var. *depressa* (from the western hemisphere). The historical introduction of *J. c.* var. *communis* is discussed as well as a morphological key to *J. communis* varieties. Published on-line www.phytologia.org *Phytologia 98(1):8-16 (Jan 5, 2016).* ISSN 030319430.

KEY WORDS: *Juniperus communis* var. *communis*, *J. c.* var. *depressa*, introduced species, DNA, sequence, petN-psbM.

The genus *Juniperus* is diverse with approximately 67 species (Adams, 2014). *Juniperus communis* is the only species that grows in both eastern and western hemispheres. It is composed of approximately 10 varieties, with 5 in the eastern and 5 in the western hemisphere (Adams, 2014).

Genetic analysis of Arctic populations of *J. communis* (Adams et al., 2003) revealed that these populations clustered by continent, with the populations in Greenland and Iceland showing the highest affinities to populations from Europe and not to those from North America. Adams et al. (2003) concluded that the post-Pleistocene populations on Greenland and Iceland came from Europe and not North America. Adams and Pandey (2003) analyzed *J. communis* and its varieties by use of RAPDs and found considerable variation, but several of the varieties were not discernable.

Adams and Nguyen (2007) collected additional samples of putative *J. c.* var. *saxatilis* from the Pacific northwest, *J. c.* var. *jackii* from NW California and *J. c.* var. *depressa* from the southernmost locations in North America (Mt. Charleston, Nevada and Mt. Satula, North Carolina). They found the major trend among the taxa was the separation of the eastern hemisphere plants (*J. communis* var. *communis, J. c.* var. *saxatilis,* and putative *J. c.* var. *saxatilis*, Kamchatka) from the western hemisphere plants *(J. c. var. depressa*, *J. c.* var. *jackii*, *J. c.* var. *megistocarpa*, and putative *J. c.* var. *saxatilis*). The resolution of *J. c.* var. *jackii* (and plants from Mt. Hood) was in contrast to the report by Ashworth, et al. (1999, 2001).

Adams (2008) examined nrDNA SNPs in varieties of *J. communis* in North America and found *J. c.* var. *jackii* (now *J. jackii* (Rehder) R. P. Adams) to be very distinct along with the juniper from Queen Charlotte Island (recognized as *J. c.* var. *charlottensis* R. P. Adams). Interestingly, *J. c.* var. *depressa* and *J. c.* var. *saxatilis* (Japan) were found to be identical in their nrDNA.

Most recently, Adams et al. (2011) examined *J. communis* and related species by nrDNA plus four cp regions. They found a complex pattern of differentiation among the *communis* varieties (Fig. 1).

Notice (Fig. 1) that *J. c.* var. *kamchatkensis* is more closely allied with var. *kelleyi* (nw USA) than var. *saxatilis* (Japan). *Juniperus c.* var. *communis* (Sweden, Armenia) are quite distant from the North American taxa (Fig. 1).

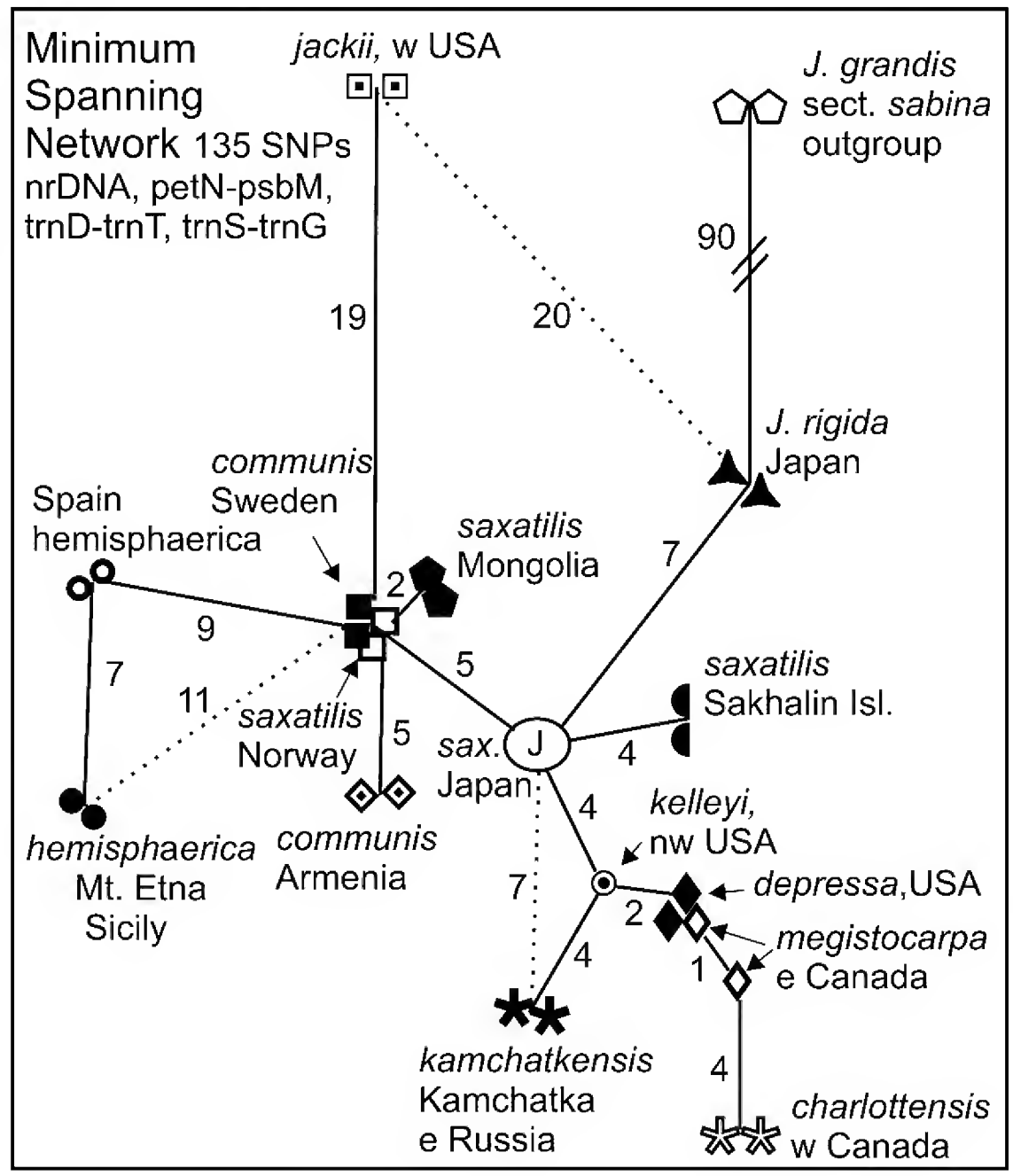

Figure 1. Minimum spanning network based on 135 SNPs. Modified from Adams et al. (2011).

One of the earliest report of putative *J. communis* (var. *communis*) as a tree in New England was by Terry (1901) who examined a population described by Rehder in Cyclopedia of American Horticulture (1900). The population (Terry, 1901) was near Northampton, MA in which trees were "erect, some of them columnar, others spreading" in the "same field with the common prostrate form, *J. communis canadensis* Loud." (now treated as *J. communis* var. *depressa* Pursh). Fernald (1950) also mentions upright *J. communis* trees in New England.

Examination of a *J. communis* var. *depressa* population near Cushman, MA, about 8 mi east of Northampton revealed that several plants had one or several upright stems ranging up to 4 m tall (*Adams 8569-8571*, MATERIALS AND METHODS). Continued searches for single stemmed *J. communis* trees have seen unsuccessful by the senior author until recently when his colleagues of this study shared information on upright *J. communis* trees they had seen in New England and West Virginia. This study is

the result of collaboration between these authors in supplying materials of *J. communis* from upright trees in Maine, Massachusetts, Vermont and West Virginia.

Rossbach (1963) described *J. communis* near Helvetia, Randolph Co., WV: "these obvious columnar trees, though wild, are presumably escapes from the yards and cemeteries of the local Swiss farmers...". Bartgis and Hutton (1988) reported another population of *J. communis* trees in Pendleton Co. WV as well as Highland Co., VA (T. Wieboldt, pers. comm.) and stated that these populations were native. Recent efforts to relocate the Pendleton Co. population were unsuccessful (BS and JV). Prior to this molecular study, all specimens of *J. communis* from Helvetia and Pendleton Co. at West Virginia University Herbarium (WVA) were annotated as J. *communis* var. *communis* based on leaf characters (JV).

Juniperus communis and varieties from the eastern hemisphere are distinguished from *J. communis* varieties in the western hemisphere by a few mutations in the cp region petN-psbM (Adams et al., 2011). The purpose of this study was to examine the petN-psbM region by DNA sequencing to determine if any of the upright tree *J. communis* trees in New England and West Virginia are in fact *J. c.* var. *communis* from Europe or merely upright sports of *J. c.* var. *depressa* (from the western hemisphere).

MATERIALS AND METHODS

Maine:

Art Gilman 07229, Lab Acc *Adams 14506*, upright tree, 2-3 m, under power line, small popn. on flood terrace, on N side of Austin Stream, 45° 04' 10.8" N; 69° 52' 59" W, 600 ft, Bingham, Somerset Co., ME, 28 Sep 2007;

Massachusetts:

Adams 8569, 4m shrub/tree, some stems vertical, some horizontal, 1.7 mi E of Cushman,MA, on Flat Hill Rd. 0.25mi N of Jct. Flat Hills Rd. & Shutesbury Rd., then 400ft E on private road. 42° 24' 24.22"N, 72° 29' 36.42"W, 427 ft., Hampshire Co., MA, 7 Aug 1998;

Adams 8570, 4m shrub/tree, some stems vertical, some horizontal, 1.7 mi E of Cushman,MA, on Flat Hill Rd. 0.25mi N of Jct. Flat Hills Rd. & Shutesbury Rd., then 400ft E on private road. 42° 24' 24.22"N, 72° 29' 36.42"W, 427 ft., Hampshire Co., MA, 7 Aug 1998;

Adams 8571, 2.5 m tall x 2 m wide shrub, other typical shrubs of J. c. var. depressa in the population, 1.7 mi E of Cushman,MA, on Flat Hill Rd. 0.25mi N of Jct. Flat Hills Rd. & Shutesbury Rd., then 400ft E on private road. 42° 24' 24.22"N, 72° 29' 36.42"W, 427 ft., Hampshire Co., MA, 7 Aug 1998;

Hickler ns, Lab Acc. *Adams 14549*, upright tree, 1.5 m tall, female, on Charlemont Isl. in Deerfield River, 1 mi. east of Charlemont, or 2 mi nw of Shelburne Falls, 42° 37' 13" N; 72° 46' 18" W, 477 ft, Franklin Co., MA, 17 May 2015;

Vermont:

Art Gilman 05002 and *E. C. Briggs* Lab Acc *Adams 14507*, single large tree in open pasture, Rice Hill Rd., Hartland, near Vermont, New Hampshire border. 43° 32' 12" N; 72° 24' 52" W, 690 ft, Windsor Co., VT, 29 Apr 2005;

West Virginia:

Brian P. Streets 5462, Lab Acc. *Adams 14503*, Otter Creek Wilderness Area, Monongahela Nat. For., sedge fen, w *Picea rubens*, *Rubus hispidus*, *Carex*. 38° 58' 53.29" N; 79° 39' 36.22" W, 3250 ft, Randolph Co., WV, 4 Nov 2014;

Brian P. Streets 5463, Lab Acc. *Adams 14504*, Helvetia, WV, ca. 0.2 air mi. from jct CR 45 and CR46. in CR 46 ditch-line, overhung by oak forest. 38° 42' 15.87" N; 80° 11' 52.56" W, 2240 ft, Randolph Co., WV, 14 Jan 2015;

Brian P. Streets 5543 Lab Acc *Adams 14509*, Lower Glady, farm off Sulley Rd (CR 1/2), near cemetery, s of Three Springs Run, in old field. 38° 57' 17.47" N; 79° 37' 07.09" W, 2690 ft, Randolph Co., WV, 25 Feb 2015;

Jim Vanderhorst 7892 Lab Acc *Adams 14510*, Lower Glady, farm off Sulley Rd (CR 1/2), near cemetery, s of Three Springs Run, in old field. 38° 57' 17.47" N; 79° 37' 07.09" W, 2690 ft, Randolph Co., WV, 25 Feb 2015;
Voucher specimens are deposited at the Baylor University herbarium (BAYLU).

One gram (fresh weight) of the foliage was placed in 20 g of activated silica gel and transported to the lab, thence stored at -20° C until the DNA was extracted. DNA was extracted from juniper leaves by use of a Qiagen mini-plant kit (Qiagen, Valencia, CA) as per manufacturer's instructions.

Amplifications were performed in 30 µl reactions using 6 ng of genomic DNA, 1.5 units Epi-Centre Fail-Safe Taq polymerase, 15 µl 2x buffer E (petN, trnD-T, trnL-F, trnS-G) or K (nrDNA) (final concentration: 50 mM KCl, 50 mM Tris-HCl (pH 8.3), 200 µM each dNTP, plus Epi-Centre proprietary enhancers with 1.5 - 3.5 mM $MgCl_2$ according to the buffer used) 1.8 µM each primer. See Adams, Bartel and Price (2009) for the ITS and petN-psbM primers utilized. The primers for trnD-trnT, trnL-trnF and trnS-trnG regions have been previously reported (Adams and Kauffmann, 2010).

The PCR reaction was subjected to purification by agarose gel electrophoresis. In each case, the band was excised and purified using a Qiagen QIAquick gel extraction kit (Qiagen, Valencia, CA). The gel purified DNA band with the appropriate sequencing primer was sent to McLab Inc. (San Francisco) for sequencing. Sequences for both strands were edited and a consensus sequence was produced using Chromas, version 2.31 (Technelysium Pty Ltd.) or Sequencher v. 5 (genecodes.com). NJ (Neighbor Joining analysis was the MAFFT analysis package (http://mafft.cbrc.jp/alignment/server/).

RESULTS AND DISCUSSION

Sequencing petN-psbM yielded 717 bp. NJ analysis using petN-psbM data shows that the Cushman, MA; Hartland, VT and Deerfield River, MA plants group with *J. communis* from the western hemisphere (boldface, Fig. 2).

However, Bingham, ME; Otter Ck., WV; and Lower Glady, WV group with *J. communis* of the eastern hemisphere (boldface Fig. 2). This supports the thesis that these plants are escaped cultivars of *J. c.* var. *communis*.

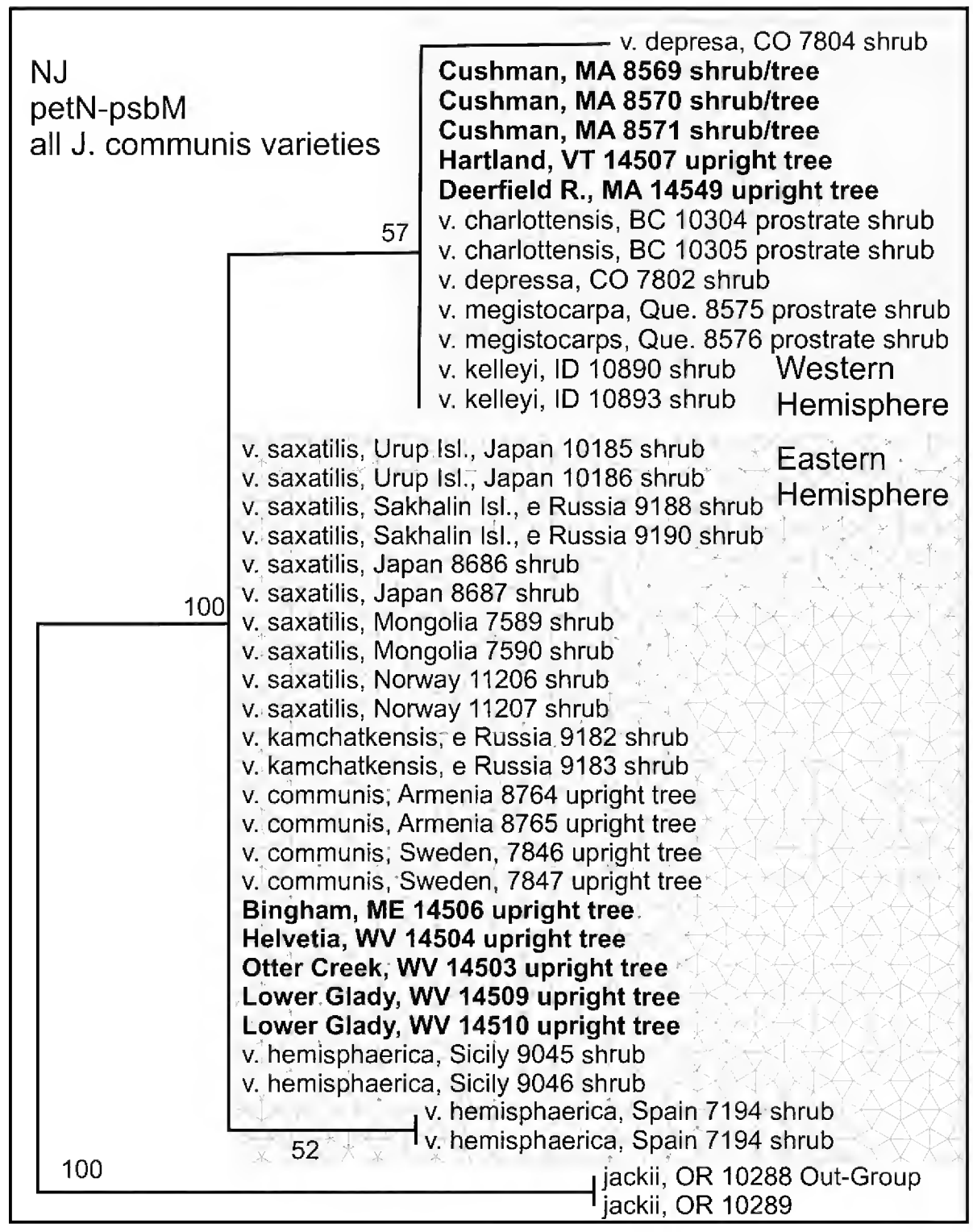

Figure 2. NJ clustering of US *J. communis* trees with all other varieties of *J. communis* using petN-psbM sequence data.

A point mutation is present at position 533 in the petN-psbM sequence. The nucleotide is an A for Cushman, MA, Hartland, VT and Deerfield River, MA and all the *J. communis* varieties in the western hemisphere (Table 1). Position 533 is a T for Helvetia, Otter Creek, and Lower Glady, WV as well as Bingham, ME and all *J. communis* varieties in the eastern hemisphere (Table 1). This provides evidence that these *communis* trees are from seed from cultivated *J. c.* var. *communis* trees.

Table 1. Nucleotide substitutions and indels in *J. communis* from eastern and western hemispheres. Upright shrubs and trees from northeastern US are in **boldface**.

From Western Hemisphere	position 533	positions 541-545(5 bp indel)
Cushman, MA 8569 upright shrub	**A**	- - - - -
Cushman, MA 8570 upright shrub	**A**	- - - - -
Cushman, MA 8571 upright shrub	**A**	- - - - -
Hartland, VT 14507 upright tree	**A**	- - - - -
Deerfield R., MA 14549 upright tree	**A**	- - - - -
All native w hemi. varieties of J. communis:		
var. Charlottensis, BC 10304 prostrate shrub	A	- - - - -
var. Charlottensis, BC 10305 prostrate shrub	A	- - - - -
var. depressa, Colorado 7802 shrub	A	- - - - -
var. megistocarpa 8575 prostrate shrub	A	- - - - -
var. megistocarpa 8576 prostrate shrub	A	- - - - -
var. depressa, Colorado 7804 shrub	A	- - - - -
var. kelleyi, Idaho, 10890 shrub	A	- - - - -
var. kelleyi, Idaho, 10893 shrub	A	- - - - -
From Western Hemisphere but like Eastern Hemisphere *J. communis* var. *communis*		
Helvetia, WV 14504 upright tree	**T**	- - - - -
Bingham, ME 14506 upright tree	**T**	- - - - -
Otter Creek, WV 14503 upright tree	**T**	**CTTCT**
Lower Glady, WV 14509 upright tree	**T**	**CTTCT**
Lower Glady, WV 14510 upright tree	**T**	**CTTCT**
From Eastern Hemisphere, all J. communis varieties:		
var. communis, Sweden, 7846 upright tree	T	- - - - -
var. communis, Sweden, 7847 upright tree	T	- - - - -
var. communis, Armenia 8764, upright tree	T	- - - - -
var. communis, Armenia 8765, upright tree	T	- - - - -
var. hemispherica, Sicily 9045, shrub	T	- - - - -
var. hemispherica, Sicily 9046, shrub	T	- - - - -
var. hemispherica, Spain 7194, shrub	T	- - - - -
var. hemispherica, Spain 7195, shrub	T	- - - - -
var. kamchatkensis, e Russia 9182, shrub	T	- - - - -
var. kamchatkensis, e Russia 9183, shrub	T	- - - - -
var. saxatilis, Urup Isl., Japan 10185, shrub	T	- - - - -
var. saxatilis, Urup Isl., Japan 10186, shrub	T	- - - - -
var. saxatilis, Sakhalin Isl., e Russia 10188, shrub	T	- - - - -
var. saxatilis, Sakhalin Isl., e Russia 10190, shrub	T	- - - - -
var. saxatilis, Japan 8686, shrub	T	- - - - -
var. saxatilis, Japan 8687, shrub	T	- - - - -
var. saxatilis, Mongolia 7589, shrub	T	- - - - -
var. saxatilis, Mongolia 7590, shrub	T	- - - - -
var. saxatilis, Norway 11206, shrub	T	- - - - -
var. saxatilis, Norway 11207, shrub	T	- - - - -

Figures 3-8 show verified *J. c.* var. *communis* from West Virginia and Maine.

Fig. 3. var. *communis*, Otter Creek, WV

Fig. 4. Habitat, var. *communis*, Otter Creek, WV

Fig. 5. v. *communis*, close-up, Otter Creek, WV.

Fig. 6. v. *communis*, close-up, Lower Glady, WV

Fig. 7. var. *communis*, Bingham, ME

Fig. 8. Google Earth 'street view' of power-line near Bingham, ME. Arrow points possible *J. c.* var. *communis* tree.

Juniperus c. var *depressa*, although normally a shrub, can be a small tree in New England. Figures 9-11 show trees in Hartland, VT (Fig. 9), Cushman, MA (Fig. 10) and Charlemont Island, Deerfield River, MA (Fig. 11).

It is interesting that the three trees from WV all contain an indel (CTTCT) not found in any other varieties (Table 1). It seems likely that these trees arose from seed of a single parent (or group of siblings) that also have the indel. The *J. c.* var. *communis* trees at Lower Glady and Helvetia, WV appear to have originated from seed dispersed by birds (Adams and Thornburg, 2010) from var. *communis* cultivated at nearby homes and cemeteries (Fig. 12). Columnar trees such as *Cupressus sempervirens, Juniperus chinensis* (strict cultivars) and *J. c.* var. *communis* (strict trees) are popular plantings in cemeteries in the US.

Fig. 9. var. *depressa* tree, Hartland, VT

Fig. 10. var. *depressa*, note 2 upright stems, Cushman, MA

Fig. 11. var. *depressa*, Charlemont Isl., Deerfield River, MA

Fig. 11. Elkins, WV cemetery with cultivated *J. c.* var. *communis* trees.

The Otter Creek, WV population of *J. c.* var. *communis* consists of hundreds to thousands of trees of various sizes in a remote Wilderness Area. Although it is less than 3 air miles from the Lower Glady site and could have originated from seed transported from this site by birds, it is also possible that it was planted in Otter Creek prior to Wilderness designation during the logging boom around 1900. In any case, the population is robust and expanding in wetland habitat, to the extent that it may displace native vegetation at the site.

As an aid for the identification of *communis* trees, a key to *communis* varieties in North America is presented (including var. *communis*, an escaped, cultivated tree in the ne US).

1a. seed cones 10 – 13 mm diam., much larger than leaf length, known only from southeastern Canada.....var. ***megistocarpa***
1b. seed cones 6 – 9 mm diam., smaller or about equal to leaf length, other than se Canada
2a. glaucous stomatal band about as wide to 1.5 x as wide as each green leaf margin, prostrate or low shrub with ascending branchlet tips (or occasionally a spreading shrub), leaves upturned (to 15 mm), rarely spreading, linear to curved, rarely a small, strict tree (to 2-4 m) in the New England.....var. ***depressa***
2b. glaucous stomatal band twice or more as wide as each green leaf margin, upright shrubs or spreading, mat-like shrubs or introduced strict trees (var. *communis*)
3a. glaucous stomatal band twice or more as wide as each green leaf margin, boat-shaped, curved leaves, mature seed cones length greater than leaf length, spreading, mat-like shrub, grows in muskeg bogs, Calvert Island to Queen Charlotte Islands, and north to, Chichagof Island, Alaska.....var. ***charlottensis***
3b. glaucous stomatal band 2,3, 4 times as wide as each green leaf margin
4a. strict (columnar) trees, leaves long (15-20 (30) mm), straight (not curved), stomatal band 2 - 3 x as wide as green leaf margin.....(escaped cult. in ne US).....var. ***communis***
4b. shrubs in western US, leaves short (<15 mm), curved, stomatal band 3-4 x as wide as each green leaf margin,
5a. gland on brown sheath elongated oval or if a long narrow gland, then with a rounded bottom end, immature seed cones globose, leaves most straight to slightly curved, not usually boat-shaped, free (not appressed to stem or leaf above on branchlet), usu. shrubs to 0.5 m tall with upturned to elevated branchlets, not on serpentine, but grows various habitats from granite, sandstone, alluvial, sand, and lava.....**var. *kelleyi*** (prev. treated as var. saxatilis)
5b. gland on brown sheath long, narrow, raised; immature seed cones elongated-subglobose, leaves curved, boat-shaped, appressed to stem or leaf above on branchlet; shrubs, usually prostrate or mat-like on serpentine or ultramafic rock (sometimes on volcanic lava, rarely on granite.....***J. jackii*** (included in this key as it is often confused with var. *kelleyi*)

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Comparison of leaf terpenoids and tannins in *Juniperus osteosperma* from woodrat (*Neotoma stephensi*) browsed and not-browsed trees

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ABSTRACT

Neotoma lepida (woodrat) browses on the leaves of *Juniperus osteosperma* near Dugway, UT. A comparison between woodrat (*N. lepida*) browsed and not-browsed *Juniperus osteosperma* trees revealed that the percentage of total volatile leaf oil yields was not significantly different between browsed trees (2.22%, 24 hr dist., DM-basis) and not-browsed trees (2.47%). On a percent total oil basis, α-pinene (4.5, 3.0%) was highly significantly higher in browsed trees, while α-campholenal (1.1, 1.3%) was significantly higher in not-browsed trees. On a mg/g DW basis, α-campholenal (0.23, 0.33%) and four compounds [p-cymene (0.34, 0.57), sabina ketone (0.20, 0.30), terpinen-4-ol (1.74, 2.67) and p-mentha-1,4-dien-7-ol (0.17, 0.25)] were significantly higher in not-browsed trees. There was a trend (P=0.075) for protein-precipitable phenolics (PPP) concentrations to be lower (3.64 mg/ g) in browsed than not-browsed (7.68 mg/ g). There was also a trend (P=0.081) for nitrogen content to be higher in browsed (0.76%) than not-browsed (0.67%). ADF (acid detergent fiber) was non-significant and averaged 27.33%. Published on-line www.phytologia.org *Phytologia 98(1): 17-25 (Jan. 5, 2016).*

KEY WORDS: *Juniperus monosperma, Neotoma stephensi,* woodrats, browsing, terpenes, protein-precipitable phenolics (PPP), nitrogen, ADF (acid detergent fiber) diet selection.

Populations of *Neotoma lepida* in the Great Basin utilize *J. osteosperma* for both food and shelter (Stones & Hayward 1968). Recent evidence suggests that one population in White Rocks Utah may actually specialize on *J. osteosperma.* with fecal pellet analysis showing >90% of plant fragments present to be *J. osteosperma* (unpublished observation, M. Skopec). Juniper foliage is visible in midden entrances (Fig. 1) and evidence of herbivory is present on many trees in the area (Fig. 2). However, the removal of foliage is non-random from adjacent trees (Fig. 2), suggesting that the woodrats are making foraging decisions, perhaps avoiding trees high in terpenes, similarly to another pine specialist, *Sciurus abert* (Abert's squirrel, Snyder 1992) or phenolics. *Neotoma stephensi,* a closely related specialist on *J. monosperma,* shows a similar foraging style on juniper and analysis of the terpene profiles of browsed and not-browsed junipers revealed that only one terpene, p-cymene, was found in higher concentration in not-browsed compared to browsed junipers, suggesting that *N. stephensi* is making foraging decisions based not on avoiding high levels of terpenes but perhaps seeking out higher nutrient content, or closer proximity to middens (Adams et al. 2014a). While much analysis of *N. stephensi's* physiological adaptations that allow it to metabolize the terpenes present in *J. monosperma* have been done (Boyle & Dearing, 2003; Dearing, McLister, & Sorensen, 2005; Haley, Lamb, Franklin, Constance, & Dearing, 2007; McLister, Sorensen, & Dearing, 2004; Skopec & Dearing, 2011; Skopec, Haley, & Dearing, 2007; Sorensen, Turnbull, & Dearing, 2004; Torregrossa, Azzara, & Dearing, 2011) very few studies have been conducted on mechanisms that *N. lepida* may utilize for terpene metabolism (Magnanou, Malenke &

Dearing, 2009; Skopec, Malenke, Halpert & Dearing, 2013; Wilderman et al., 2014). If analysis of browsed versus not-browsed *J. osteosperma* for differences in terpene and nutrient content reveal that *N. lepida* does not avoid terpenes like *N. stephensi,* more detailed analysis of *N. lepida* physiological mechanisms for metabolizing terpenes may be warranted.

Figure 1. Midden entrance. Note juniper leaves at the entrance to the midden/

Fig. 2. Not-browsed (left) and browsed (right) *J. osteosperma* trees near woodrat middens in Utah.

Considering the amount of research on the specialist woodrat (*N. lepida*), it is surprising that we could find no publication concerning the composition of *J. osteosperma* leaves from browsed trees vs. not-browsed trees. Although it should be noted that Adams (1994, 2012, 2013a, 2013b) and Adams and Kauffmann (2010) have published several studies of geographic variation in the leaf essential oils of *J. osteosperma* and on the effects of grinding leaves (Adams et al. 2014b). The purpose of this paper is to present new data on leaf volatile oils, protein-precipitable phenolics (PPP), nitrogen (N) and acid detergent fiber (ADF) from *J. osteosperma* leaves from *N. lepida* browsed and not-browsed trees.

MATERIALS AND METHODS

Plant material: *Juniperus osteosperma, Adams 14291-14300,* browsed trees, *Adams 14301-14310,* not-browsed trees, all common on and near granite, at White Rocks natural area, 7.4 mi n of Jct UT 199 and UT 196, thence 8 mi. w of UT 196. ~16 mi (25.7 km) nw of Dugway, UT, *40 19.367' N, 112 53.924' W*, 5254 ft (1567 m), 28 May 2014. Herbarium vouchers are deposited in the herbarium, Baylor University (BAYLU).

Essential oils analysis - A portion (200 g FW) of the fresh foliage was kept cool (20ºC) and in the dark, then, exhaustively steam-distilled for 24 h using a modified circulatory Clevenger-type apparatus (Adams 1991). Oil samples were concentrated (diethyl ether trap-removed) with nitrogen and stored at -20ºC until analyzed. Steam distilled leaves were oven dried to a constant weight (48 hr, 100ºC) for the determination of oil yield as [oil wt./(oil wt. + oven dried extracted foliage wt.)]. The extracted oils were analyzed on a HP5971 MSD mass spectrometer: 0.2 ul of a 10% solution (in diethyl ether) oil injected, split, 1:10, temperature programmed, linear, 60º - 246ºC at 3ºC/min. (62 mins.), carrier gas He, flow 34.96 cm/sec or 1.02 ml/min, injector 220ºC, detector 240ºC, scan time 1/sec, directly coupled to a HP 5890 gas chromatograph, using a J & W DB-5, 0.26 mm x 30 m, 0.25-micron coating thickness, fused silica capillary column (see Adams 2007, p. 4, for detailed operating conditions). Identifications were made by searches of our volatile oil library (Adams 2007) using HP Chemstation library search routines, coupled with retention time data of authentic reference compounds. Quantification was by flame ionization detector on an HP 5890 gas chromatograph operated under the same conditions as the GCMS (above) using the HP Chemstation software.

Protein-Precipitable Phenolics (PPP) - Condensed tannins were purified for subsequent use as a standard from dried *J. osteosperma* leaves modifying the method described by Wolfe et al. (2008) using Sephadex LH-20 (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). Protein-precipitable phenolics (PPP) were measured according to Hagerman and Butler's (1978) scaled down method as modified to determine protein precipitability of condensed tannins in two duplicate crude plant extracts (Naumann et al., 2013).

Nitrogen determination (N) - N (X 6.25 = crude protein) concentration. Samples were assayed for N concentration by combustion using an Elementar vario Macro C:N analyzer (Elementar Americas, Inc, Mt. Laurel, NJ, USA).

Acid Detergent Fiber (ADF) - ADF was determined by methods described originally by Van Soest et al., (1991) using an Ankom 200 Fiber Analyzer (Ankom Technologies, Macedon, NY, USA).

Statistical analyses - Terpenoids (as percentage of total oil and as mg per g dry foliage weight), PPP, N, and ADF concentrations were compared between browsed and not-browsed samples by ANOVA and SNK (Student-Newman-Keuls) analyses as described by Steele and Torrie (1960). Differences were considered significant at $P \leq 0.05$, unless otherwise stated.

RESULTS AND DISCUSSION

A detailed compositional analysis of *J. osteosperma* volatile leaf oils from browsed and not-browsed trees is shown in Table 1. ANOVA of the leaf volatile oils components (% total oil basis) for browsed and not-browsed trees revealed the percentage of total volatile leaf oil yields was not significantly different between browsed trees (2.22%, 24 hr dist., DM-basis) and not-browsed trees (2.47%). The lack of a significant difference in the yields of volatile oils was surprising. However, it is instructive to compare browsing (mostly goats) on two juniper species growing in the same population. For *J. ashei*, Adams et al. (2013a) found the browsers selected for lower leaf oil yield. But, in a companion study of browsed *J. pinchotii* (in the same population with *J. ashei* in the 2013a study), Adams et al. (2013b) found no significant difference in % oil yield between browsed and not-browsed trees. The closely related juniper specialist, *N. stephensi,* also seems to not make foraging decisions based on total amount of volatile oils (Adams et al. 2014a).

On a percent total oil basis, α-pinene (4.5, 3.0%) was highly significantly different and α-campholenal (1.1, 1.3%) significantly different between browsed and not-browsed trees. On a mg/g DW basis, α-campholenal (0.23, 0.33%) was highly significantly different and four compounds [p-cymene (0.34, 0.57), sabina ketone (0.20, 0.30), terpinen-4-ol (1.74, 2.67) and p-mentha-1,4-dien-7-ol (0.17, 0.25) were significantly different. Notice that four (of five) of these terpenoids are oxygenated (alcohols, an aldehyde and a ketone). Oxygenated compounds are generally more bio-reactive than hydrocarbons. The only terpene, α-pinene, that was found to be higher in browsed trees, is the major terpene in *N. stephensi*'s preferred plant *J. monosperma*, where it is found in levels 3-4 times that in *J. osteosperma* (Adams, Skopec, & Muir 2014). It is likely that *N. lepida* is able to effectively metabolize the lower concentrations of α-pinene found in *J. osteosperma.* Also a potentially interesting idea may be that *N. lepida* is actually seeking out α-pinene as a cue for trees that are lower in the oxygenated compounds, which may be more toxic. Based on these results it seems that *N. lepida* is making foraging decisions to avoid specific terpenes present in *J. osteosperma.* This pattern of not avoiding an entire class of PSC's, but only specific potentially bioactive members of a class of PSC's has been seen in other dietary specialists like the koala and pygmy rabbit (Moore & Foley, 2005; Ulappa et al., 2014).

There was a trend for protein-precipitable phenolics (PPP) concentrations to be lower (3.64 mg/ g) in browsed than not-browsed (7.68 mg/ g) trees (Table 2). If PPP (cf. tannins) interfere with digestion or decrease palatability, selecting trees with less PPP might be favored by woodrats (Bernays, Elizabeth, Cooper-Driver, & Bilgener, 1989; Haslam, 1989). There was also a trend for nitrogen concentration to be higher in browsed (0.76%) than not-browsed (0.67%), trees (Table 2). Selecting trees with higher

nitrogen might be expected but higher nitrogen can also be a result of younger material in regrowth points (Assefa et al., 2008; Reynolds et al., 1992). ADF varied little and was non-significant (Table 2).

Table 2. Protein-precipitable phenolics (PPP), Nitrogen and Acid Detergent Figer (ADF) for leaves of *J. osteosperma* (browsed by woodrats and not-browsed), Dugway, UT. ns = not significant at P= 0.05.

	browsed	not-browsed	F ratio	F significance
Protein-precipitable phenolics (PPP)	3.64 mg/g	7.68 mg/g	3.497	P = 0.075 ns
Nitrogen	0.76 %	0.67 %	3.337	P= 0.081 ns
Acid detergent fiber (ADF)	27.05 %	27.61 %	0.568	P= 0.533 ns

Principal coordinates (PCO)using 12 terpenes (mg/g) and oil yield (mg/g) data revealed an interesting pattern (Fig. 3). The trees appear to be in two groups, but not all browsed or not-browsed trees are in one group. Trees that were heavily browsed (Fig. 2, dashed line on right) are readily recognized. And even light browsing on a tree can be easily identified by the approximately 45° angle of the branchlet cut. It is likely, however, that trees may be lightly browsed on the top, and this browsing not visible from the ground. Thus, some trees are likely classed as not-browsed, when in fact they are being browsed (note four not-browsed trees within the dashed line ellipse with browsed trees, Fig. 3). In addition, it seems possible that a few trees may be sampled by woodrats and the cut branch discarded because it does not meet the woodrat's selection criteria (note one browsed tree within solid line ellipse with not-browsed trees, Fig. 3).

It is tempting to re-classify the trees based on oils and re-analyze the statistics, but that is not statistically valid. Greater attention to field identification of browsed and not-browsed trees may resolve this issue. Unfortunately, the trees sampled were not tagged, so we can not reexamine the trees in the field. Another difficulty in collecting was the lack of not-browsed trees in the area near the largest middens. Thus, it was necessary to move away from the midden(s) to find enough trees that were 'not-browsed'. If we inadvertently got out of the home range of the woodrats, some of the 'not-browsed' trees may not have been subject to browsing selection by woodrats. Male and female *N. lepida* were found to move only 252 and 136 ft on average from their middens a night in a similar habitat (Stones & Hayward, 1968).

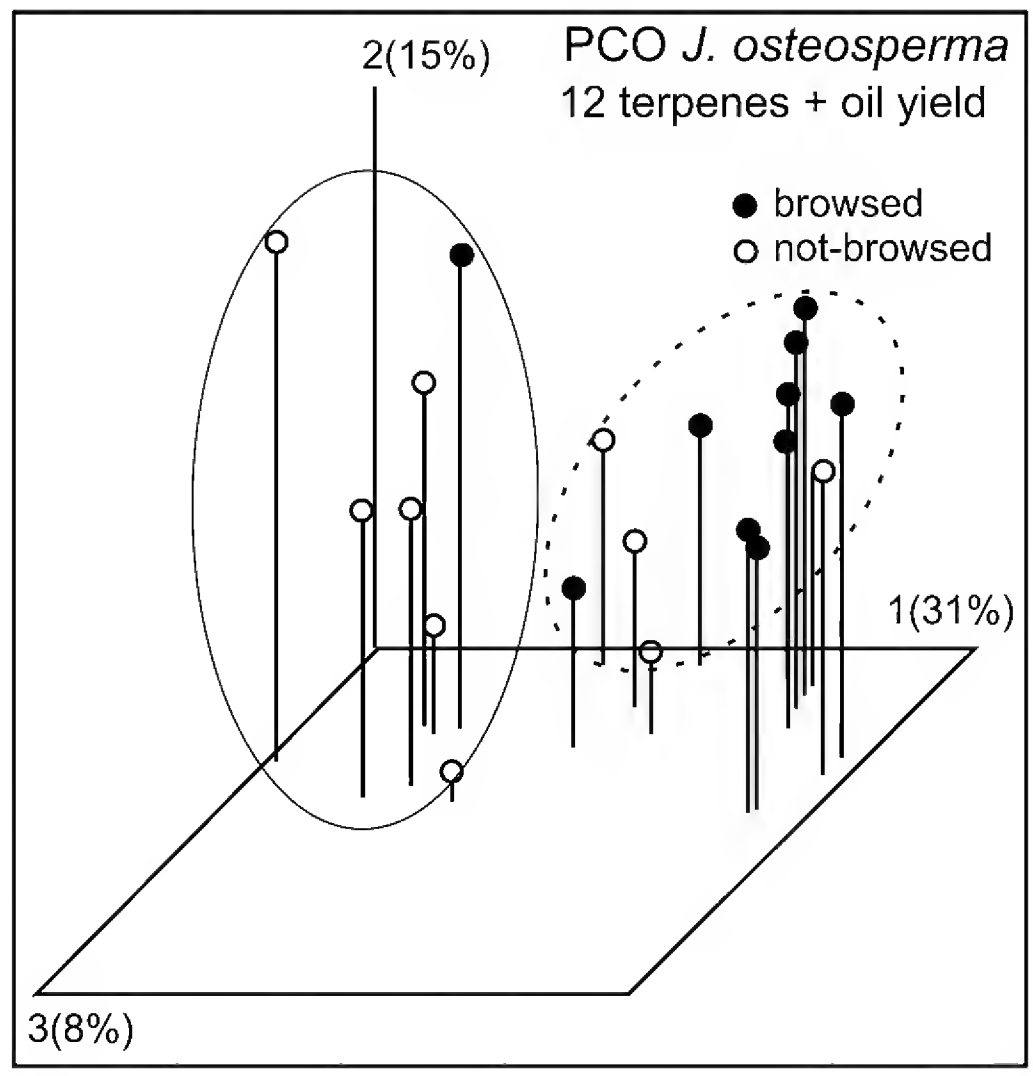

Figure 3. PCO of *J. osteosperma* trees browsed and not-browsed by woodrats. Ordination based on 12 terpenes (mg/g) and oil yield (mg/g) with character matches weighted by {[square root (F+1)]-1}. Where F is from ANOVA between browsed (10) and not-browsed (10) trees.

Compared to *N. stephensi*, that did not make foraging decisions based on terpene or tannin content, *N. lepida* seems to be choosing plants lower in oxygenated compounds and tannins and higher in α-pinene and protein (Adams et al., 2014a). While nutrient content of *J. monosperma* browsed by *N. stephensi* has not been measured based on results here and other studies with dietary specialists it is likely that *N. stephensi* do make foraging decisions based on nutrient density of the foliage (Moore & Foley, 2005; Schmalz, Wachocki, Wright, Zeveloff & Skopec, 2014; Ulappa et al., 2014).

In summary, analyses of browsed and not-browsed *Juniperus osteosperma* trees revealed that the percentage of total volatile leaf oil yield was lower, but not significantly different between browsed trees (2.22%, 24 hr dist., DM-basis) and not-browsed trees (2.47%). On a percent total oil basis, α-pinene (4.5, 3.0%) was significantly higher and α-campholenal (1.1, 1.3%) significantly lower in browsed versus not-browsed trees. On a mg/g DW basis, α-campholenal (0.23, 0.33%) and four compounds [p-cymene (0.34, 0.57), sabina ketone (0.20, 0.30), terpinen-4-ol (1.74, 2.67) and p-mentha-1,4-dien-7-ol (0.17, 0.25)] were significantly higher in not-browsed trees. There was also a trend for protein-precipitable phenolics (PPP) to be lower (3.64 mg/ g,7.68 mg/ g) and nitrogen concentration to be higher in browsed (0.76%) than not-browsed (0.67trees. ADF varied little and was non-significant. Taken together, it seems that *N. lepida* are making foraging decisions based on avoidance of PSM's and maximizing nitrogen intake.

ACKNOWLEDGEMENTS

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Table 1. Leaf essential oil compositions (% total oil basis and mg/g basis) for *J. osteosperma* (browsed and not-browsed), Dugway, UT. * = P 0.05, ** = P 0.001, ns = not significant at P= 0.05.

KI	Compound	browsed % total oil	not-browsed % total oil	F ratio, signif.	browsed mg/g	not-browsed mg/g	F ratio, signif.
	% oil & mg/g yield	2.22 %	2.47 %	1.48 ns	22.2	24.7	1.48 ns
846	(2E)-hexenal	0.3 %	0.3 %	nt	0.07	0.07	nt
921	tricyclene	0.5	0.6	nt	0.11	0.14	nt
924	α-thujene	0.3	0.3	nt	0.07	0.07	nt
932	**α-pinene**	**4.5**	**3.0**	**8.11** **	0.98	0.76	2.61 ns
946	camphene	0.6	0.6	nt	0.13	0.14	nt
953	thuja-2,4-diene	0.2	t	nt	0.04	t	nt
969	sabinene	5.4	5.3	0.19 ns	1.17	1.27	0.18 ns
974	β-pinene	0.1	t	nt	0.02	t	nt
988	myrcene	1.2	0.9	2.52 ns	0.26	0.23	0.55 ns
1002	α-phellandrene	0.2	0.2	nt	0.04	0.04	nt
1014	α-terpinene	1.0	1.2	0.57 ns	0.22	0.28	2.18 ns
1020	**p-cymene**	1.6	2.5	2.42 ns	**0.34**	**0.57**	**5.98** *
1024	limonene	2.5	2.0	3.34 ns	0.56	0.49	0.61 ns
1025	β-phellandrene	1.7	1.9	0.62 ns	0.38	0.48	2.06 ns
1044	(E)-β-ocimene	t	t	nt	t	t	nt
1054	γ-terpinene	1.6	1.9	0.68 ns	0.36	0.46	2.56 ns
1065	cis-sabinene hydrate	0.9	1.0	0.00 ns	0.21	0.24	0.46 ns
1067	cis-linalool oxide	t	t	nt	t	t	nt
1078	camphenilone	t	t	nt	t	t	nt
1086	terpinolene	0.9	0.8	0.05 ns	0.19	0.20	0.48 ns
1098	trans-sabinene hydrate	1.2	1.3	0.09 ns	0.27	0.31	0.74 ns
1102	isopentyl-isovalerate	t	t	nt	t	t	nt
1112	3-me-3-buten-me-butanoate	0.3	t	nt	0.07	t	nt
1118	cis-p-menth-2-en-1-ol	t	t	nt	t	t	nt
1122	**α-campholenal**	**1.1**	**1.3**	**6.21** *	**0.23**	**0.33**	**14.27** **
1141	camphor	21.9	21.7	0.01 ns	5.19	5.52	0.09 ns
1141	verbenol	11.0	11.1	0.00 ns	2.60	2.80	0.14 ns
1145	camphene hydrate	1.8	1.3	2.10 ns	0.38	0.33	0.94 ns
1154	**sabina ketone**	0.9	1.2	2.13 ns	**0.20**	**0.30**	**4.42** *
1160	pinocarvone	0.2	0.1	nt	0.04	t	nt
1165	borneol	4.5	5.3	0.86 ns	0.93	1.38	3.41 ns
1174	**terpinen-4-ol**	8.1	11.4	2.07 ns	**1.74**	**2.67**	**5.47** *
1179	p-cymen-8-ol	0.8	0.9	0.77 ns	0.18	0.22	1.83 ns
1186	α-terpineol	0.6	0.6	0.04 ns	0.12	0.14	1.93 ns
1195	myrtenol	0.2	0.2	nt	0.04	0.05	nt
1204	verbenone	1.6	1.2	1.67 ns	0.33	0.30	0.34 ns
1215	trans-carveol	1.6	1.3	1.05 ns	0.33	0.33	0.00 ns
1219	coahuilensol, me-ether	0.3	t	nt	0.07	t	nt
1223	citronellol	t	t	nt	t	t	nt
1226	cis-carveol	0.4	0.3	nt	0.09	0.07	nt
1238	cumin aldehyde	0.3	0.4	nt	0.07	0.09	nt
1239	carvone	0.8	0.8	0.92 ns	0.18	0.19	0.04 ns
1283	α-terpinen-7-al	t	t	nt	t	t	nt
1284	bornyl acetate	10.0	8.6	0.43 ns	2.19	2.15	0.01 ns
1298	carvacrol	0.6	0.5	1.25 ns	0.14	0.11	0.48 ns
1325	**p-mentha-1,4-dien-7-ol**	0.8	1.1	1.79 ns	**0.17**	**0.25**	**5.21** *
1468	pinchotene acetate	0.4	0.2	nt	0.08	0.05	nt
1513	γ-cadinene	t	t	nt	t	t	nt

KI	Compound	browsed % total oil	not-browsed % total oil	F ratio, signif.	browsed mg/g	not-browsed mg/g	F ratio, signif.
1522	δ-cadinene	t	t	nt	t	t	nt
1548	elemol	1.1	0.9	0.42 ns	0.23	0.23	0.01 ns
1574	germacrene-D-4-ol	t	t	nt	t	t	nt
1582	caryophyllene oxide	t	t	nt	t	t	nt
1627	1-epi-cubenol	t	t	nt	t	t	nt
1630	γ-eudesmol	t	t	nt	t	t	nt
1644	epi-α-muurolol	t	t	nt	t	t	nt
1649	β-eudesmol	t	t	nt	t	t	nt
1652	α-eudesmol	t	t	nt	t	t	nt
1652	α-cadinol	t	t	nt	t	t	nt
2312	abieta-7,13-diene-3-one	t	t	nt	t	t	nt

KI = linear Kovats Index on DB-5 column. Compositional values less than 0.1% are denoted as traces (t). Unidentified cpds. less than 0.5% are not reported.

Melampodium elottianum (Asteraceae: Heliantheae) A new species from along the Rio Cuixmala, Jalisco

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ABSTRACT

A novel taxon, **Melampodium elottianum** B.L. Turner, **sp. nov.,** is described from along the Rio Cuixmala of western Jalisco. It presumably belongs to the Sect. Serratura. A photograph of the Type is provided, along with a key to the Mexican species of the Sect. Serratura, including distribution maps. Published on-line www.phytologia.org *Phytologia 98(1): 26-29 (Jan 5, 2016).* ISSN 030319430.

KEY WORDS: Asteraceae, Heliantheae, *Melampodium*, Mexico, Jalisco

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Routine identification of Mexican Asteraceae has revealed the following novelty, a low elevation riparian species from along the Rio Cuixmala of the Chamela Bay Region, Jalisco.

MELAMPODIUM ELOTTIANUM B.L Turner, **sp. nov. Fig. 1**

Annual herbs to 30 cm high. **Mid-stems** glabrous or nearly so. **Leaves** opposite, 3-4 cm long, 1.0-1.5 cm wide; petioles 1-4 mm long, passing into the blades; blades lanceolate, pinnately veined, sparsely pubescent above and below, the margins entire. **Heads** single, terminal or axillary, 5-6 mm wide, 4-5 mm high, the ultimate peduncles sparsely pubescent, 2-4 cm long. **Involucres** of 5 broadly ovate bracts, 2-3 mm long, 1-2 mm wide, pubescent below with stiff hairs, their margins not membranous. **Receptacles** ca 2 mm wide, 3 mm high; pales linear-oblanceolate their apices rounded, pubescent. **Ray florets** 11, fertile; ligules "yellow," ca 3 mm long, 2 mm wide, under surfaces with 3-6 prominent green ribs. **Achenes** somewhat arcuate, epappose, glabrous, ca 2 mm long, having 3 prominent lateral ribs and a prominent dorsal rib. **Disk florets** ca 30, sterile, the corollas yellow, glabrous.

TYPE: **MEXICO. JALISCO: Mpio. La Huerta,** "Rancho Cuixmala, W of the Puerto Vallarta--Barra de Navidad (Mex 200) hwy., along the Rio Cuixmala." 19 23 N, 104 58 45 W, "Riparian zone. Uncommon straggling perennial," 12 Jan 1991, *Emily J. Lott 3188* [with B.L. Phillips] (Holotype: TEX).

As noted above, the collectors described the plant as a straggling perennial, but it appears to be a tap-rooted annual, to judge from its root system. Lott (1993), in her checklist of the region concerned, listed the type as **M. microcephalum**, this presumably my misidentification at the time.

In McVaugh's treatment of **Melampodium** for Flora Novo-Galiciana, the novelty will key, reluctantly, to, or near, **M. glabrum**, a poorly known species of aquatic habitats that Stuessy (1972, 1979) positioned in the Sect. Alcina, (along with **M. nutans** and **M. perfoliatum)**. Stuessy et al. (2011), using DNA data, treated **M. nutans** and **M. glabrum** as belonging to 2 newly established, monotypic sections. Their studies also suggested that Sect. Serratura was a natural grouping.

Melampodium elottianum appears to belong to the Sect. Serratura of **Melampodium** (Stuessy 1972; Stuessy et al. 2011); in Mexico, the complex contains six species, including the widespread, very common, **M. divaricatum** (Map 1) and the relatively rare taxa, **M. dicoelocarpum**, **M. tepecense** and the very rare **M. sinaloense** from NW Mexico, **M. northingtonii** from Oaxaca (Turner 1988) and the

presently described **M. elottianum** (Map 2), the latter presumably a riparian species of low elevations in western Jalisco, as noted on the type itself.

The novelty is named for Emily Lott, long time student of The Mexican flora and author of the checklist of the Chamela Bay Region of Jalisco (Lott 1993).

Artificial key to the Mexican taxa of Sect. Serratura

1. Ligules of ray florets 3.5-7.0 mm long; widespread.....**M. divaricatum**
1. Ligules of ray florets 1-3 mm long; western Mexico...(**2**)

2. Ultimate peduncles mostly 15-75 mm long...(**4**)
2. Ultimate peduncles mostly 0-15 mm long...(**3**)

3. Ligules of ray florets 0.8-1.0 mm long; Nay, Jal, Col, Mic..... **M. tepicense**
3. Ligules of ray florets 1.5-2.0 mm long; n Sin..... **M. sinaloense**

4. Involucres 3-6 mm high; lateral surfaces of achene having 2 deep oval pits; peduncles mostly 30-70 mm long.....**M. dicoelcarpum**
4. Involucres 2-3 mm high; lateral surfaces of achene otherwise; peduncles mostly 8-40 mm long; Jal, Oax... (**5**)

5. Petioles 5-8 mm long; ray florets 5; Oax.....**M. northingtonii**
5. Petioles 1-4 mm long; ray florets 11; Jal .....**M. elottianum**

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Thanks to Emily Lott for bibliographic information and to Jana Kos for editorial assistance. My Academic son, Prof. Tod Stuessy (Turner 2015), attempted DNA analysis from leaf material of the holotype for phyletic purposes, but was unable to obtain meaningful data. I much appreciated his attempt to do so. My reexamination of the type concerned still strongly suggests (to me at least) that it relates to **M. northingtonii**, which Stuessy et al. (2011) position in the Sec. Serratura.

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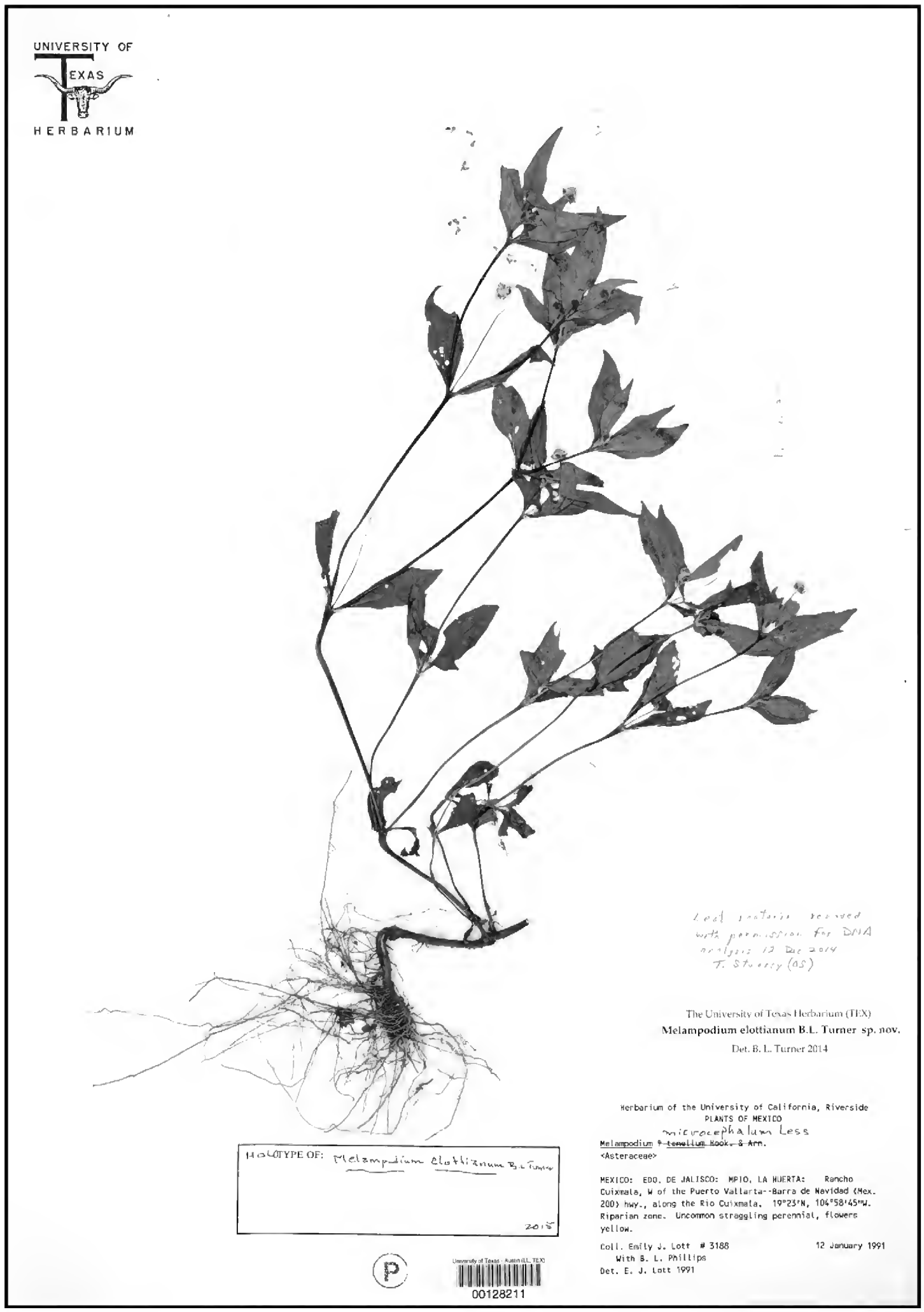
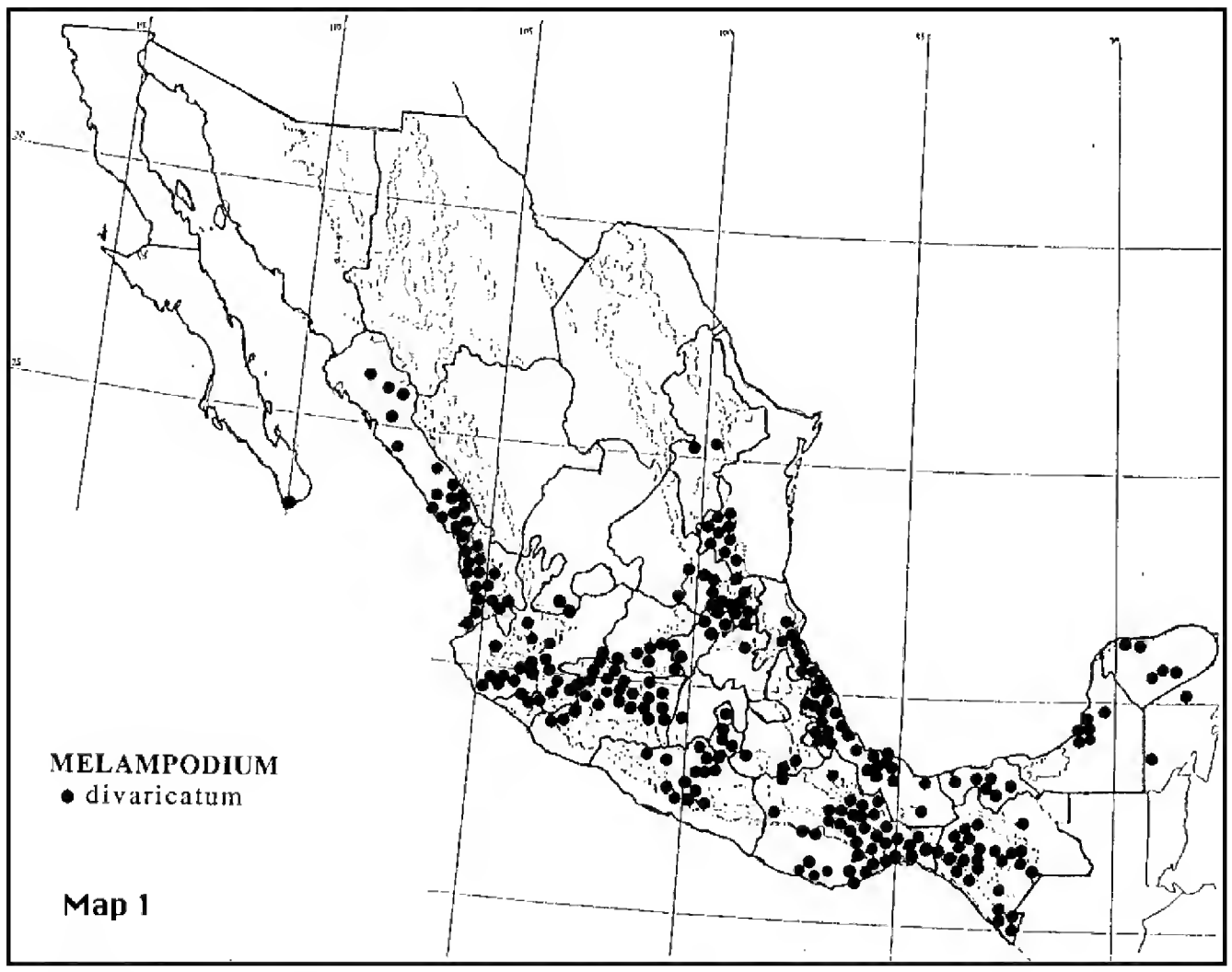
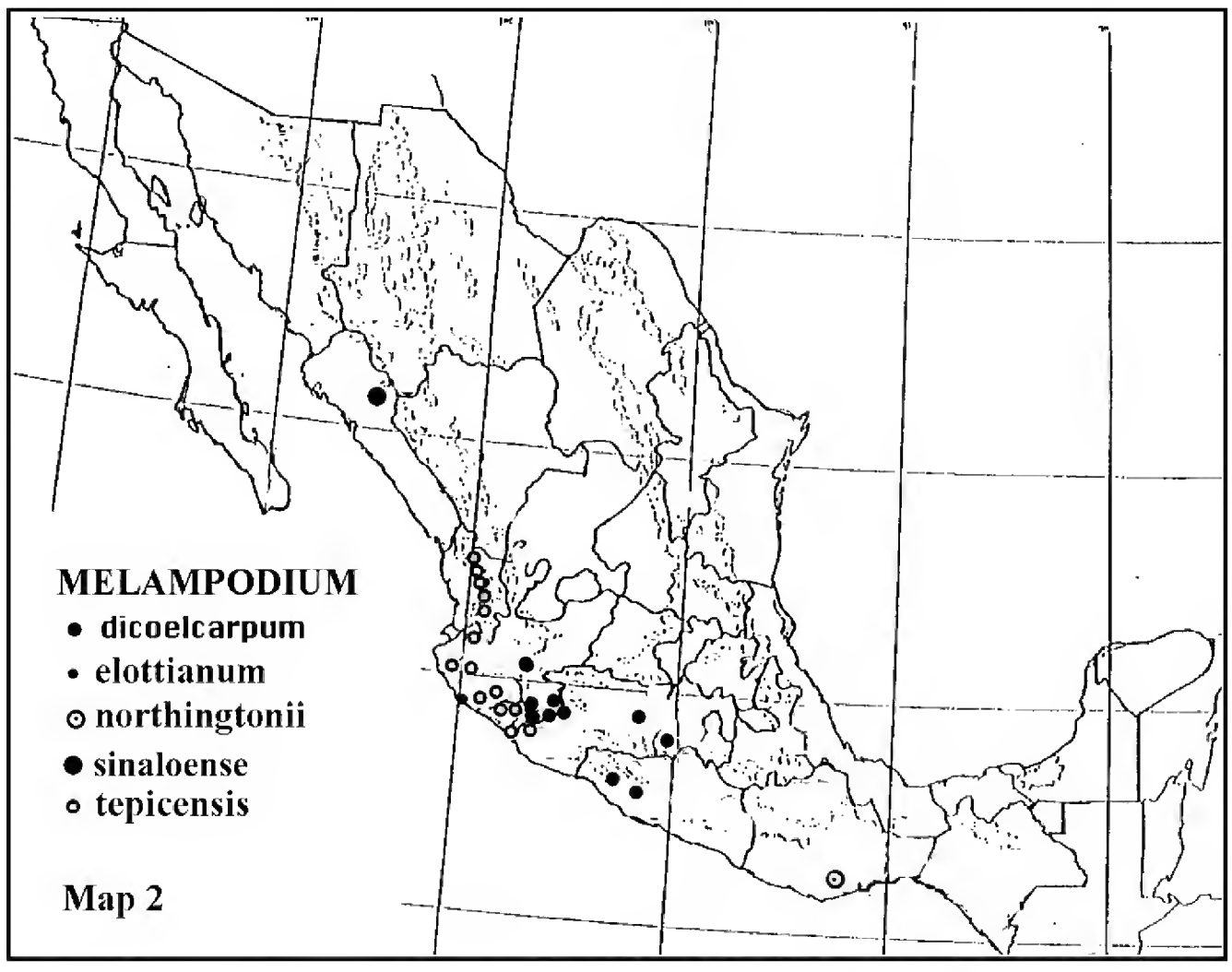


Figure 1. *Melampodium elottianum*


MELAMPODIUM
• divaricatum

Map 1


MELAMPODIUM
• dicoelcarpum
• elottianum
⊙ northingtonii
● sinaloense
⚬ tepicensis

Map 2

Survey of non-polar extractables (bio-crude) from *Grindelia ciliata* (ASTERACEAE: Astereae)

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ABSTRACT

Non-polar (pentane) extractables (bio-crude) were examined from natural populations of *Grindelia ciliata* from Texas, Oklahoma and Kansas. The highest yielding populations were from ne Amarillo, TX (14.79%), Childress, TX (13.53%) and Bullock Rd., Newcastle, TX (12.84%). The lowest yielding populations were from Spearman, TX (6.78%) and Cimarron River, KS (6.72%). The most variable populations were: Lake Tanglewood (LT, 20.7%), Childress (CAT, 19.56%) and Waco, TX (WCO, 18.6%). The populations with least variation were: Borger, TX (BOR, 5.5%), Lamesa, TX (LAM, 7.6%) and McGregor, TX (McG, 8.8%). The yields from 166 individuals screened ranged from 3.89 to 18.92%. The top five percent of the plants (8) ranged from 15.76 to 18.92%, indicating a good pool of plants for improvement of yields. The optimum time for maximum bio-crude yields was not determined, and appears to vary by elevation and length of growing season. Published on-line www.phytologia.org *Phytologia 98(1): 30-36 (Jan 5, 2016).* ISSN 030319430.

KEY WORDS: *Grindelia ciliata,* Asteraceae, non-polar extractables, bio-crude, geographic variation, Spanish Gold, giant gumweed.

Grindelia (gumweed) is a large genus of about 75 species with an amphitropical distribution with half of the species occur in North America and Mexico and the remaining species in South America (Moore, et al., 2012). Steyermark (1934) recognized 45 species, plus 40 varieties and 25 forms (110 taxa). Strother and Wetter (2006) estimated the genus to contain some 30 species; they recognized only 18 species in the Flora of North America. Bartoli and Tortosa (2012) recognized 41 species, 10 varieties and 2 forms in North America based on classical morphology. Moore et al. (2012) utilized nrDNA and the ETS region as well as psaI-accD cpDNA to analyze selected taxa of *Grindelia* from both North and South America. They found strong support for two sister clades in North and South America. The North American clade seemed to be divided into two groups by the continental divide.

Nesom, Suh and Simpson (1993) submerged the monotypic genus *Prionopsis* (*P. ciliata*) into *Grindelia* as *G. ciliata* (Nuttall) Sprengel. [syn: *G. papposa* (Nutt.) Nesom & Suh]. *Grindelia ciliata* reportedly grows as an annual or biennial species. It is widely distributed in Texas, Oklahoma and Kansas, se Colorado, e New Mexico, s Nebraska, s and se Iowa (rare) with putative outlying records from Illinois, Missouri, Arkansas and Louisiana. Distribution reports from Arizona and California are

problematic. Seinet (2015) maps a record (*G. papposa* = *G. ciliata*) in nw Michigan, but the specimen is *Jeff Hansen sn,* Horsethief Canyon, Ellsworth, KS, 30 Sep 2009 and is obviously mis-mapped.

Grindelia has been the subject of several studies on its use as a 'bio-crude' species (Adams et al. 1986; Hoffmann and McLaughlin, 1986; McLaughlin and Linker, 1987) and the genus is well known for its diterpene acids (Timmermann et al., 1987) which can be used as a substitute for pine resins in the plastics and paper industries as well, as cracked and re-formed for use as fuel (bio-fuel). Much of the work by the Arizona group was based on *G. camporum* Greene, native to central California. McLaughlin and Hoffmann (1982) reported cyclohexane (non-polar) yields from: *G. aphanactis* (= *G. nuda* var. *aphanactis*): 7.2%; *G. camporum*: 5.5 - 13.0%; *G. robusta*: 8.4%; and *G. squarrosa*: 8.8 - 13.8%. Adams et al. (1986) reported hexane (non-polar) yields from: *G. acutifolia* (CO): 5.0 - 10.23%; *G. aphanactis* (NM): 7.95%; *G. arizonica* var. *stenophylla* (CO): 8.36%; *G. decumbens* (CO): 7.11%; *G. fastigiata* (CO): 7.47 - 9.62%; *G. nana* (WA): 10.36%; *G. squarrosa*: ND - 9.65%, WA - 9.04 - 11.67%, NM - 10.23%, CO - 7.16 - 11.12%; and *G. subalpina* var. *erecta* (CO): 8.62%.

Grindelia ciliata is a large plant (up to 2 m), that grows in disturbed sites in various soils and precipitations (Figs. 1, 2). It appears to have potential as a semi-arid land bio-crude crop plant. In contrast to most *Grindelia* species, in *G. ciliata*, the leaves and buds are not gummy or with exuded resin, yet, the bio-crude yields are comparable to sticky or gummy *Grindelia* species. Sequestering the resin inside the leaves would seem to be an important agronomic character, as sticky leaves would be difficult to swath and bale. The purpose of the present paper is to survey non-polar (pentane) yields from individuals in natural populations of *G. ciliata* to evaluate its potential as a source of bio-crude.

Fig. 1. Close-up of *G. ciliata* flower and buds. Grimes Co., TX, Photo by Nathan Taylor.

Fig. 2. *G. ciliata* population on Bullock Rd., Newcastle, TX. Photo by Judy Etling.

MATERIALS AND METHODS

Fresh leaves and specimens of *G. ciliata* were collected from the following populations:

1. LT, Lake Tanglewood, Amarillo, TX 35° 04' 35" N, 101° 47' 24" W, 3596 ft. at telephone tower, most plants branched, in prairie sod (buffalo grass), loam soil. 13 Aug 2015, LTP1-10,
2. SP, Spearman, Tx, 36° 11' 16" N, 100° 48' 32" W, 2949 ft, common along caliche road (Co. Rd. W), and along hwy US 83 to Canadian, TX, most plants with single stems, caliche soil on road side, 1 mi east of Jct. Tex 70 & Farm Rd. 759 on Co. Rd. W. 15 Aug 2015, SP1-10,

3. CAT, Canadian, TX, 11 mi. east of US 83 on Tex 2266. 35° 53' 22" N, 100° 02' 05" W, 2205 ft., occasional, most plants with single stems, in deep sand, lots of small black (sugar?) ants on heads. 15 Aug 2015, CAT1-10,
4. BO, 9 mi N of OK/TX border, on US83, at a ravine on terrace n of Beaver River, mostly single stemmed, locally common on sandy soil. 36° 35' 14" N, 100° 49' 42" W, 2893 ft, 19 Aug 2015, *Adams 14631*, BO1-10,
5. CK, 3 mi N of KS/OK border on KS hwy 23, n of Cimarron River, locally common, mostly single stemmed, on sandy soil. 37° 01' 27" N, 100° 29' 39.5" W, 2378 ft, 19 Aug 2015, CK1-10C,
6. DCK, on US 56, 3 mi w of jct US 56 US 54, on sandy soil in ditch, Dodge City, KS, scattered but locally common, mostly single stemmed, 37° 43' 13" N, 100° 04' 11" W, 2510 ft, 19 Aug 2015, DCK1-10,
7, 8. FH, Fritch Highway, about 17 mi ne of Amarillo, TX, 35° 25' 34" N, 101° 38' 07" W, 3520 ft. on Tex 136, most plants branched. Common on west side of hwy from this locatioin near Fritch, TX (to last ravine) on Tex 136. in prairie grass, loam soil. 21 Aug 15, recollection from same tagged plants, 3 Sept 2015, FHR1-10,
9. BOR, 1 mi s of Borger, TX on Tex 207 on road cut, sandy but caliche on top, mostly branched plants. 35° 38' 17" N, 101° 23' 50" W, 3203 ft, 22 Aug 2015. *Adams 14636*, BOR1-10,
10. AIR, few hundred plants, most single stemmed. on poor soil, mostly caliche, with sunflowers and disturbed prairie. Air Products Plant, n on Tex 136, ~1 mi s of OK border, 36° 29' 19" N, 101° 28' 20" W, 3156 ft., 1 Sept 2015, *Adams 14638*, AIR-10,
11. PST, ~5 mi w of Post, TX, on US 84 and CR 165, 20-30 plants, disturbed site, on sand, 33° 13' 02" N, 101° 26' 02" W, 2939 ft., 27 Aug 2015, PST1-5,
12. SAT, to 6 ft tall, spindly, 20-30 plants, on vacant lot, sand, on US 183 in Santa Ana, TX, 31° 44' 32" N, 99° 19' 51" W, 1725 ft., 27 Aug 2015, *Adams 14640*, SAT1-5,
13. BR, around oil tanks, on red loam, half plants were branched, on Bullock Road, near Newcastle TX, 33° 09' 34" N, 98° 41' 54" W, 1217 ft., 30 Aug 2015, *Adams 14642*, BR1-10,
14. CHD, on vacant lot in Childress, red sand, 100 plants, many branched, on US 287, 34° 24' 47" N, 100° 10' 02" W, 1737 ft, 30 Aug 2015, *Adams 14644*, CHD1-10,
15. GRU, on caliche, disturbed prairie, ~100 plants, most single stemmed, ~20% flowers seeded, Hansford Gun Club, 36° 12' 38" N, 101° 18' 59" W, 3145 ft, 1 Sept 2015. *Adams 14645*, GRU1-10,
16. McG, on vacant lot, sandy-loam, 10 plants, on US84, 7 m n of McGregor, 8 mi. s of Waco, TX, 31° 28' 48" N, 97° 17' 35" W, 540 ft., 27 Aug 2015, *Adams 14641*, MCG1-6,
17. WCO, right-of way, Tex. 3400 at Flat Creek, 4.5 miles nw of Robinson, on highly disturbed alluvium consisting of clay to silt. Disturbance is mostly due to grading to build up the edge of right-of-way to limit drainage from adjoining agricultural fields and periodic cutting. Population of about 75-100 plants spread over about 100 m (at the outer edge of right-of-way). Plants 1-1.5 m tall, probably small due to drought. 31° 29' 30.43" N, 97° 04' 51.25" W, 400 ft.,23 Aug 2015. *W. Holmes ns*, Lab. Acc. *Adams 14646*, WCO1-10,
18. LMT Lamesa, TX population, 3-4 mi. w of the Gaines-Dawson Co. line about a mile south of 180. 32°41'07" N, 102°15'51" W, elev. 3063 ft., *Nathan Taylor ns*, 20 Aug 2015, LMT1-10.

Pentane non-polar extraction - Leaves were collected from branched plants, so we could remove leaves from 4 branches without seriously damaging the health of the plant. Four side branches (each approximately 30 cm long) were cut and the fresh leaves stripped into a paper bag. Upon return to the lab, the bags were air dried (40 - 42°C) for 24 h (7.8% moisture). The leaves were ground in a coffee mill to pass a 1 mm screen. Three (3) g. of air-dried leaves were extracted with 19 ml of pentane on a platform shaker for 24 h. The supernatant was filtered into a pre-weighed aluminum disposable pan. The extracted material (marc) was washed with 2 ml of pentane and the supernatant filtering into the aluminum pan. The pentane was evaporated (40°C) in a hood. The pan, with extract, was weighed and the tare weight subtracted.

Paired extractions (3) using pentane-shaker (24 h) vs. pentane-soxhlet extraction (8h, until no color was being removed) revealed that the pentane-soxhlet extraction resulted in 2.1x higher yields than

pentane-shaker extractions. Because pentane-shaker extractions can be done in large numbers, this method was chosen and the yields corrected by a 2.1 factor. The final shaker yields were corrected to an oven dry weight (ODW) basis by post-multiplication of correction factor (CF=2.29), where CF = 2.11 (soxhlet/shaker yields) x 1.085 (air dried wt./ ODW) = 2.29.

ANOVA was computed for percent crude oil and means tested for significance using Student-Newman-Keuls (SNK) multiple range tests of significance, computed by use of program SNK, written by RPA, using formulation Steel and Torrie (1960). Pearson Product Correlation was computed by use of http://www.socscistatistics.com/tests/pearson/Default2.aspx.

RESULTS AND DISCUSSION

The yields of bio-crude varied considerably within and among populations (Table 1). The highest yielding populations were ne Amarillo (14.79%), Childress (13.53%) and Bullock Rd. (12.84%) (Table 1). The lowest yielding populations were Spearman (6.78%) and Cimarron River, KS (6.72%) (Table 1). To visualize the geographic variation, yields were contour mapped (Fig. 3). It is interesting that the highest yielding populations form a nw to se line (FH, CHD, BR). Yields in most of the other populations range from 6.72 to 9.12%, except the SAT population (10.6%). Of course, this is the product of both genes and the environment. It might be noted that this year (2015) was the 5th wettest year in recorded history at Amarillo (~32"). However, the difference between nearby populations FH (14.8%) and BOR (7.7%) seems likely genetic, and not due to changes in habitat. The populations in ne Texas Panhandle, Oklahoma and sw Kansas are all low in bio-crude. The populations from west Texas and central Texas tend to be higher in yields.

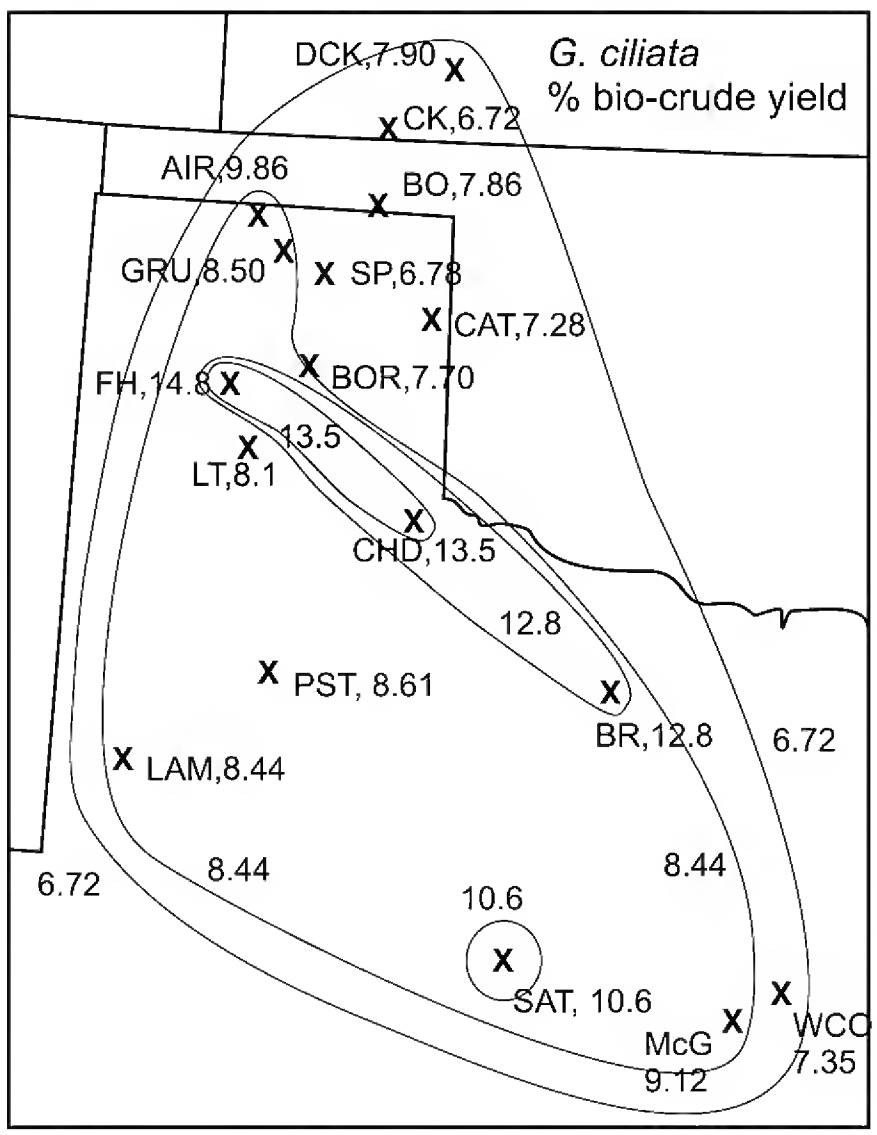

Figure 3. Contoured % bio-crude yields. Number next to the population ID is yield.

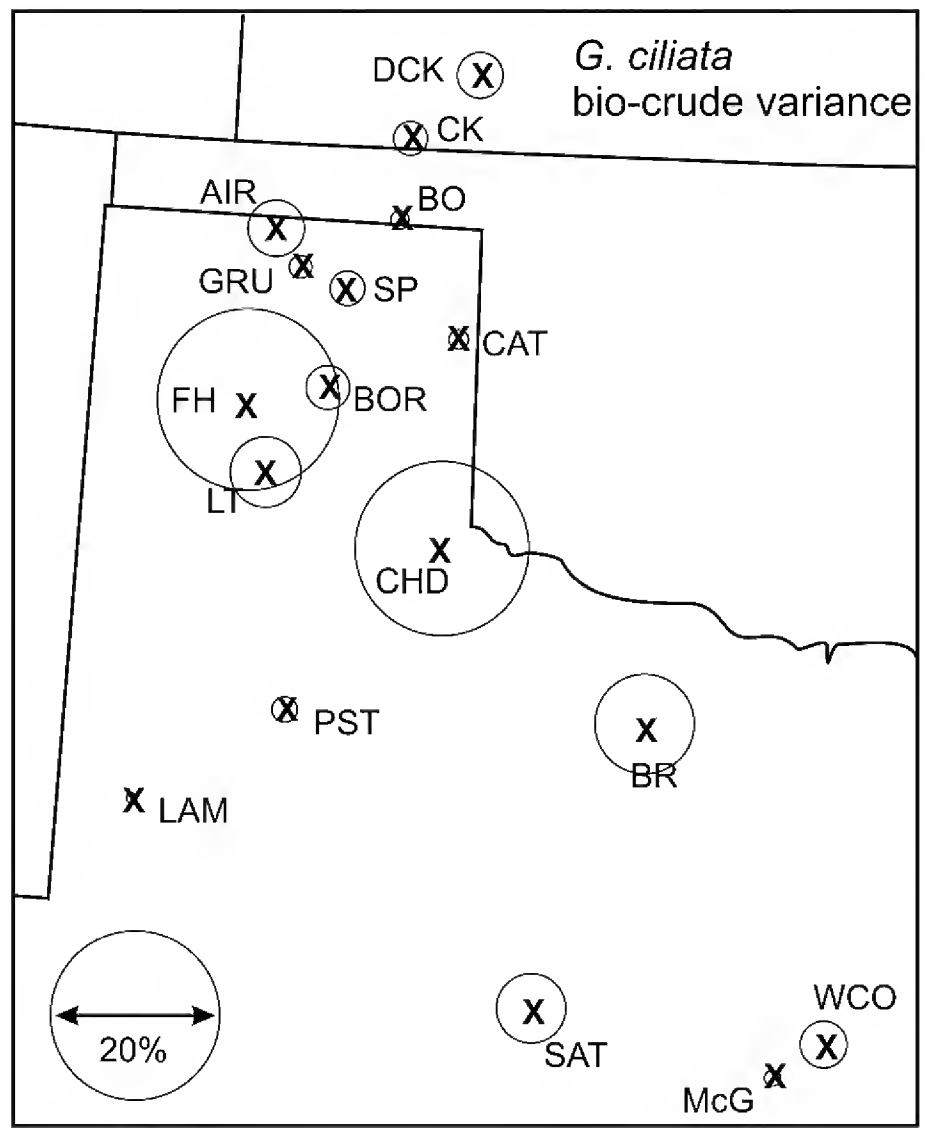

Figure 4. Coefficient of variation (%) per population. The diameters of circles are proportional to CV.

To visualized the geographic trends in population variation of bio-crude, the coefficient of variation (CV) was mapped (Fig. 4). The most variable populations were: Lake Tanglewood (LT, 20.7%), Childress (CHD, 19.56%) and Waco (WCO, 18.6%). The populations with least variation were: Borger, TX (BOR, 5.5%), Lamesa, TX (LAM, 7.6%) and McGregor, TX (McG, 8,8%).

It is instructive to compare the yields from the same plants sampled at different dates. Ten plants were tagged and leaves collected, then, later, additional leaves were collected from the same 10 tagged plants. For the ne Amarillo population (Table 2, top), the average yield dropped from 14.79% (13 Aug) to 8.07% (3 Sept). Yet, the plants were still putting on new buds and healthy. The coefficient of variation (CV) was also smaller in the later sample (Table 2, top). The correlation in yields between the two sampling dates was -0.330. Indeed, one sees essentially no correlation between yields from paired plants in table 2, top.

In contrast, yields from the Bullock Rd., Newcastle (BR) population were not significantly different between 13 Aug and 14 Sept samples (12.84 vs. 13.00%, Table 2 bottom). The CV was slightly higher in the Sept samples (15.7 vs. 17.6%, Table 2, bottom). The correlation in yields between the two sampling dates was +0.758. One sees a correlation between yields from paired plants in table 2, bottom.

It is difficult to explain these conflicting results, except to note that the ne Amarillo population (FH) is at 3,520 ft in a semi-arid grassland with dark loam, whereas Bullock Rd. (BR) is at 1217 ft. is in the rolling plains, red loam area. It may be that the bio-crude extractables have reached a maximum concentration sooner in the much shorter growing season in the high plains (FH) population. Whereas, the bio-crude yield in the longer growing season (BR) population reached a maximum later in the year. To investigate the accumulation of bio-crude throughout the growing season, we are establishing test plots in the high plains (~3,500 ft, Gruver, TX) and lower, rolling plains (~1,200 ft, Newcastle - Graham, TX).

A third comparison was made between population samples (10 plants, randomly chosen) in the Childress (CHD) population. It should be noted that we initially tagged and sampled 10 plants on 30 Aug, but upon return, to re-sample, all the tags had been removed by vandals. So we collected from 10 plants at random. Thus, in Table 3, plants 1-10 are not paired samples. Nevertheless, the averages for bio-crude yields are significantly higher in the 27 Sept samples than in the earlier, 30 Aug samples (11.30 vs. 13.53, Table 3) which is the same trend seen in the BR data (Table 2, bottom). Both of these populations (CHD, 1737 ft, BR, 1217 ft.) are off the caprock and have longer growing seasons than the ne Amarillo population (FH, 3,520 ft.). The CV for the CHD population was larger among the later (27 Sept) samples (13.8%, 19.6%, Table 3). Correlation between sampling periods could not be computed as the samples were not paired.

The distribution of the 166 plants of *G. ciliata* analyzed is shown in Fig. 5. The mode is 7.84% and otherwise normal distribution is skewed to the left. The top 5% yields range from 15.76 to 18.92% bio-crude. Although these data are from leaves, it is interesting to note that Adams et al. (1986) in a very large survey of 832 plants in 614 taxa in the western United States found only *Chrysothamnus paniculatus* and *Parthenium argentatum* with whole plant yields of 16.4%. There is clearly lots of variation in the yields of bio-crude among *G. ciliata* plants, strengthening its promise as a potential new crop for bio-crude.

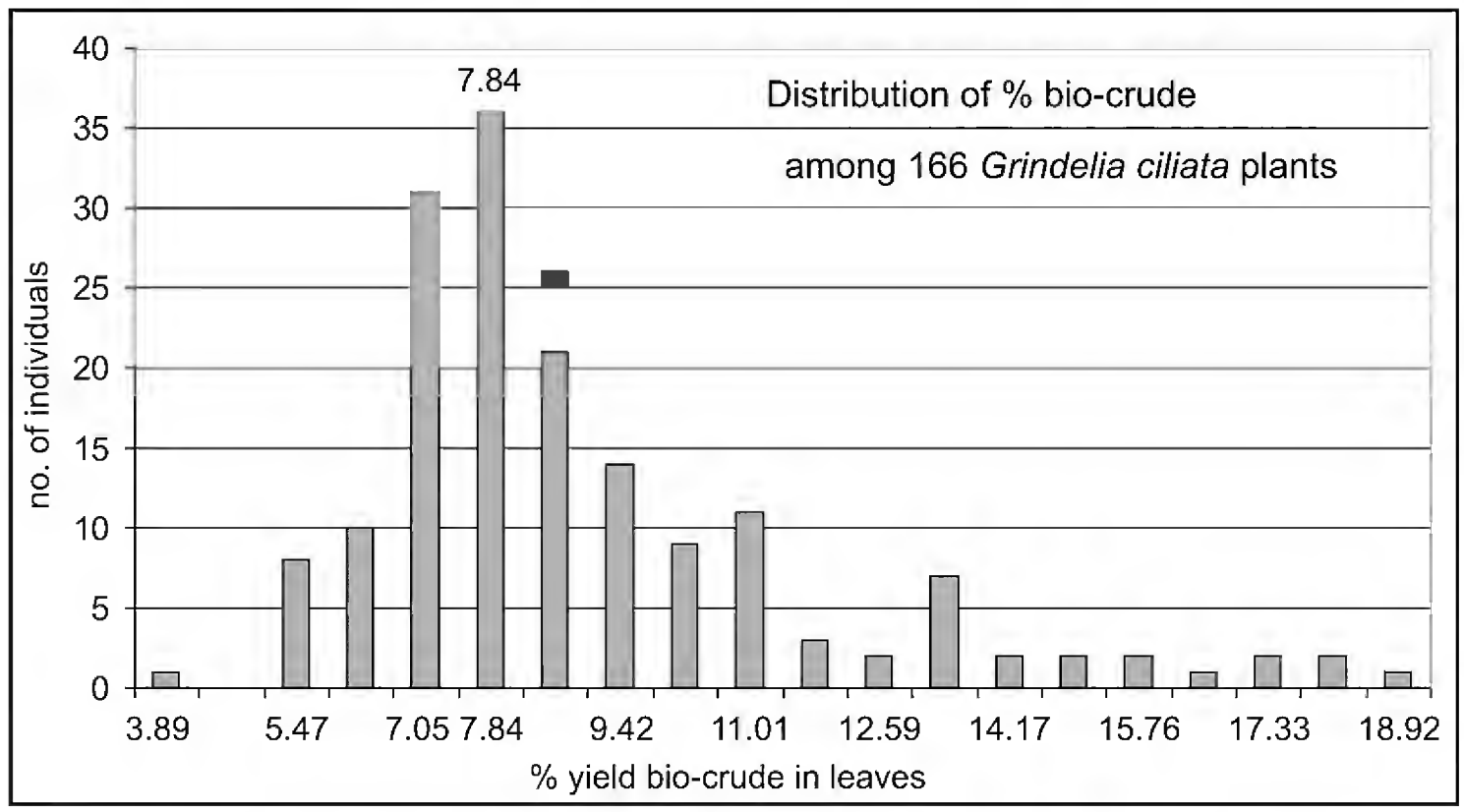

Figure 5. Distribution of % yield of bio-crude in leaves of *G. ciliata* (n= 166).

This first report on variation in bio-crude yields is preliminary because we do no know the optimum time to harvest during the growing season to obtain maximum bio-crude yields. Nevertheless, the results do support the thesis that *G. ciliata* is a promising crop for the production of bio-crude. Additional research utilizing tissue culture cloning and test plots is planned for 2016.

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Table 1. Survey of non-polar extractables (bio-crude) from *Grindelia ciliata*. Yields are as percent dry matter (DM) from leaves only. Any averages not followed by a common letter (a,b,c,d,e) are significantly different (P=0.5). CV = population covariance (%).

Population	1	2	3	4	5	6	7	8	9	10	Avg.	CV.
ne Amarillo, TX 8/13	13.88	15.58	18.28	13.65	14.61	18.92	16.98	13.84	10.71	11.43	14.79a	18.3
Childress, TX 9/27 late	13.43	10.08	15.11	12.6	16.87	18.17	13.05	9.69	13.39	12.90	13.53ab	19.6
Bullock Rd., TX	12.29	11.68	11.29	15.57	13.59	11.22	13.13	9.92	16.41	13.28	12.84b	15.7
Santa Anna, TX	7.79	12.14	11.37	11.14	10.46						10.58c	15.8
AIR Products, TX	11.14	11.14	9.16	10.15	10.61	9.84	10.99	6.71	7.97	10.94	9.87cd	15.2
McGregor, TX	9.62	8.32	9.08	8.70	8.55	10.46					9.12cd	8.8
Post, TX	8.40	8.70	10.15	8.47	7.33						8.61cde	11.7
Gruver, TX	7.86	9.54	7.33	8.54	6.87	8.63	9.47	9.80	8.32	8.63	8.50cde	11.3
Lamesa, TX	8.93	7.53	9.28	8.98	7.99	7.63	8.70	8.70	7.78	8.85	8.43cde	7.6
Lake Tanglewood, TX	8.24	9.16	8.02	10.92	10.39	7.30	6.99	7.61	5.25	7.18	8.11de	20.7
ne Amarillo, TX 9/3	7.94	6.83	7.33	9.69	10.38	7.33	7.63	7.56	8.09	7.94	8.07de	13.8
Dodge City, KS	10.70	9.42	6.56	7.00	8.78	7.29	7.67	6.93	6.63	8.03	7.90de	17.2
Beaver R., Oklahoma	6.81	6.78	7.92	7.94	8.64	7.40	9.21	7.23	7.66	9.01	7.86de	10.9
Borger, TX	7.76	8.32	7.02	7.40	7.76	7.10	7.79	7.86	8.17	7.86	7.70e	5.5
Waco,, TX	8.47	9.16	5.34	7.17	5.56	7.72	6.25	7.63	7.01	9.23	7.35e	18.6
Canadian, TX	6.34	6.41	6.77	6.95	7.79	6.34	7.02	8.08	8.53	8.55	7.28e	12.1
Spearmen, TX	6.95	6.95	7.48	7.67	3.89	5.73	7.78	7.02	7.58	6.79	6.78	17.4
Cimarron R., KS	7.25	8.85	5.65	7.51	5.59	6.30	7.48	5.56	5.46	7.56	6.72	17.4

Table 2. Comparison of non-polar extractables (bio-crude) from *Grindelia ciliata* from ne Amraillo, Tx and Newcastle, TX populations for 10 paired plants. ** = P 0.01. CV = population covariance (%).

ne Amarillo, TX 8/13/15 vs 9/3/15 samples	1	2	3	4	5	6	7	8	9	10	Avg.	CV
ne Amarillo, TX 8/13	13.88	15.58	18.28	13.65	14.61	18.92	16.98	13.84	10.71	11.43	14.79	18.3
ne Amarillo, TX 9/3	7.94	6.83	7.33	9.69	10.38	7.33	7.63	7.56	8.09	7.94	8.07**	13.8
Correlation = -0.330												

Newcastle, TX 8/13/15 vs. 9/14/15	1	2	3	4	5	6	7	8	9	10	Avg.	CV
Bullock Rd., TX 8/13	12.29	11.68	11.29	15.57	13.59	11.22	13.13	9.92	16.41	13.28	12.84	15.7
Bullock Rd., TX 9/14	11.39	10.43	10.51	15.10	14.96	11.87	16.23	10.26	14.43	14.68	13.00ns	17.6
Correlation = 0.758												

Table 3. Comparison of non-polar extractables (bio-crude) from *Grindelia ciliata* from a random samples (10 plants) collected on 30 Aug and re-sampled 27 Sept, 2015 at Childress, TX (original tags were removed by vandalism). . * = P 0.05. CV = population covariance (%).

Childress, TX (not paired samples!) 8/30/15 vs. 9/27/15	1	2	3	4	5	6	7	8	9	10	Avg.	CV
Childress, TX 8/30	10.46	11.37	10.08	8.63	13.36	11.91	11.53	9.92	13.36	12.67	11.30	13.8
Childress, TX 9/27e	13.43	10.08	15.11	12.6	16.87	18.17	13.05	9.69	13.39	12.90	13.53*	19.6
Correlation = N. A.												

Inheritance of nrDNA in artificial hybrids of *Cryptomeria japonica* cv. *Haara* and *C. japonica* cv. *Kumotooshi*

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ABSTRACT

Sequencing nrDNA of parents (*Cryptomeria japonica* cv. *Haara* and *C. japonica* cv. *Kumotooshi*) and seven artificial hybrids, revealed three of the seven (3/7) hybrids had nrDNA that was heterozygous, just as found in Kumo, whereas four of the seven (4/7) hybrids had nrDNA that was exactly like that of one parent, Haara. Sequencing petN-psbM, showed that cv. *Kumotooshi* was the male (pollen source) for all of the hybrids. The inheritance of nrDNA was not correlated with pollen source. If these results can replicated, this appears to raise a cautionary flag on the use of nrDNA in detecting hybridization. Published on-line **www.phytologia.org** *Phytologia 98(1): 37-41 (Jan. 5, 2016).* ISSN 030319430.

KEY WORDS: *Cryptomeria japonica* cv. *Haara, C. japonica* **cv.** *Kumotooshi,* hybrids, inheritance, nrDNA, petN-psbM.

Sequencing of nrDNA spacer regions has been an important source of phylogenetic information in plant systematics for several years. The conserved nature of the multi-copy nrDNA (thousands of copies per cell) might be due to concerted evolution (Liao, 1999). Liao (1999) argues that because rRNAs are structural molecules, multiple gene copies are necessary to supply the demand for ribosomal subunits in the cell. Because these sub-units function only when assembled into a large complex, homogeneity of rRNAs is critical for regular, functional ribosome assembly and translation to function normally. Liao (1999) concludes that "a possible biological function of concerted evolution is to maintain homogeneous gene copies in a family so that homogeneous transcripts can be produced." However, concerted evolution is thought to be a slow process over numerous generations. Hybrids would seem likely to be heterozygous for both parents nrDNA. Thus, nrDNA (ITS) is often used for the analysis of hybridization. Recently, Adams (2015a,b) found that nrDNA detected 15 hybrids, whereas, maldehy, a single copy nuclear gene (SCN) detected 25 hybrids. nrDNA appeared more often to be the same as one of the parents, whereas the SCN gene (maldehy) was heterozygous indicating the plant(s) were of hybrid origin.

Chaing et al. (2001) found that in the artificial hybrids between *Begonia aptera* (pollen) and *B. formosana* (maternal), nrDNA was predominantly that of the maternal parent, *B. formosana* (diamonds, Fig. 1). Volkov, et al. (1999) reported that one of the parental nrDNAs was eliminated in the allopolyploid genome of cultivated tobacco. Fukuoka et al. (1994) found that the nrDNA in γ-ray irradiated tetraploid rice was homogenized in a short time.

Aguilar et al. (1999) made artificial hybrids between *Armeria villosa* ssp. *longiaristata* and *A. colorata*, then examined the inheritance of nrDNA in F_1 and F_2 generations. They found the expected additive pattern in polymorphisms for five of the six variable sites in F_1 plants. However, in the F_2

generation, there was a bias towards one parent (*A. colorata*). Backcrosses showed homogenization of five of the polymorphic sites to the recurrent parent.

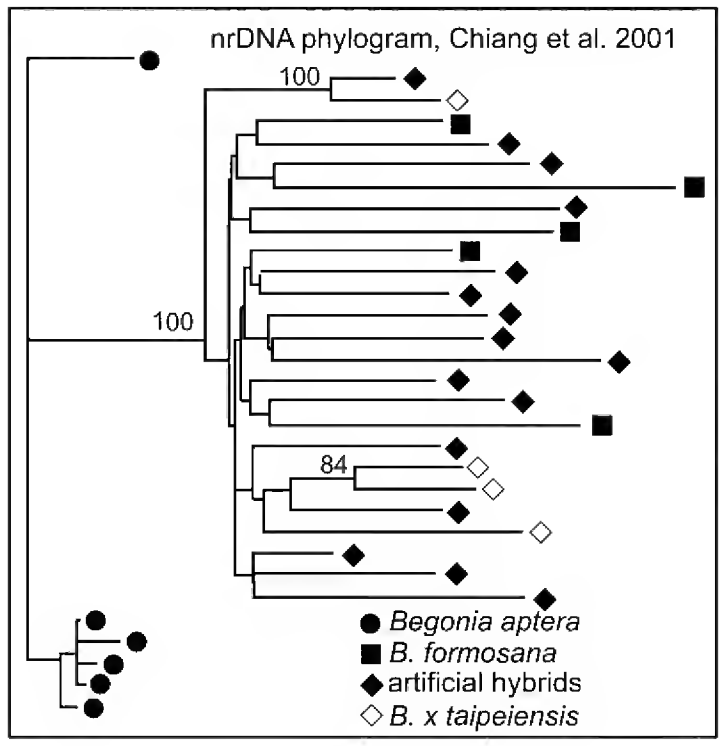

Figure 1. Phylogram based on nrDNA for *Begonia* and hybrids (adapted from Chiang, et al. 2001). Notice the grouping of the hybrids (triangles, nrDNA) with the maternal parent, *B. formosana* (shaded squares), rather than with the pollen (paternal) parent *(B. aptera*, shaded circles).

Okuyama et al. (2005) examined introgression in *Mitella* using nrDNA ITS and ETS, and cpDNA and found that cpDNA revealed the most introgression, ITS regions showed a moderate amount of introgression and the ETS region gave no evidence of introgression. They concluded that non-uniform concerted evolution between the ETS region and ITS regions may explain these different patterns of introgression.

Cryptomeria japonica D. Don (Sugi) is a monotypic genus (Farjon, 2005; Tsumura, 2011), endemic to Japan. Farjon (2005) argues that *C. fortunei* Hooibr. is conspecific, and a study (Kusumi et al. 2000) based on DNA sequencing, found no support for the recognition of *C. fortunei* separate from *C. japonica*. *Cryptomeria japonica* appears to have been introduced into China many years ago (Farjon, 2005) and is now widely cultivated in Japan, Taiwan, Korea, China and the Azores Islands (Tsumura, 2011). It is a very important commercial forest tree in Japan and the object of many detailed studies (see review, Tsumura, 2011) at the Forestry and Forest Products Research Institute and other institutes in Japan. Recently, Adams and Tsumura (2012) reported on the inheritance of leaf terpenoids from artificial hybrids of *C. japonica* cv. *Haara* X *C. japonica* cv. *Kumotooshi*. These hybrids were developed as part of a forestry improvement program at the Forestry and Forest Products Research Institute and other institutes in Japan.

In the Cupressaceae, breeding programs are rare, so the existence of parents and artificial (verified) hybrids is an important resource for studies on inheritance. This program afforded an unusual opportunity to examine the inheritance of nrDNA in hybrids in the Cupressaceae. As far as known to the authors, there are no reports on the inheritance of nrDNA in the Cupressaceae (or in conifers). The purpose of this paper is to report on the inheritance of nrDNA in artificial hybrids of *C. japonica* cv. *Haara* X *C. japonica* cv. *Kumotooshi*.

MATERIALS AND METHODS

Plant material: Crosses were made at the Forestry and Forest Products Research Institute and other institutes in Japan. Local cultivars of sugi (*Cryptomeria japonica*): 'Haara 4' (female parent) and 'Kumotooski' (male parent) were crossed and produced one hundred (100) progeny. Leaves were collected from individual hybrids 23,48,56, 65, 70, 81 and 83, growing at an outdoor nursery at the Institute. (Note: there are several clones of Haara at the Institute, so clone 4 is 'Haara 4'). Recently, the parent trees used in this cross died, but DNA was preserved. Leaf and DNA materials:

Parents: 14517 *Cryptomeria japonica* cv. *Haara*, 'Haara clone 4', DNA
14518 *C. japonica* cv. *Kumotooshi*, DNA

Seven (7) Hybrids (leaves in silica gel) (lab accession # - hybrid #):
14519 - 23, 14520 - 48, 14521 - 56, 14522 - 65, 14523 - 70, 14524 - 81, 14525 - 83.

Voucher specimens are deposited at the Dept. of Forestry and Forest Products Research Institute, Tsukuba, Ibaraki, 305-8687, Japan

One gram (fresh weight) of the foliage was placed in 20 g of activated silica gel and transported to the lab, thence stored at -20° C until the DNA was extracted. DNA was extracted from juniper leaves by use of a Qiagen mini-plant kit (Qiagen, Valencia, CA) as per manufacturer's instructions. Amplifications were performed in 30 µl reactions using 6 ng of genomic DNA, 1.5 units Epi-Centre Fail-Safe Taq polymerase, 15 µl 2x buffer E (petN-psbM), D (maldehy) or K (nrDNA) (final concentration: 50 mM KCl, 50 mM Tris-HCl (pH 8.3), 200 µM each dNTP, plus Epi-Centre proprietary enhancers with 1.5 - 3.5 mM $MgCl_2$ according to the buffer used), 1.8 µM each primer. See Adams, Bartel and Price (2009) for the ITS and petN-psbM primers utilized. The PCR reaction was subjected to purification by agarose gel electrophoresis. In each case, the band was excised and purified using a Qiagen QIAquick gel extraction kit (Qiagen, Valencia, CA). The gel purified DNA band with the appropriate sequencing primer was sent to McLab Inc. (San Francisco) for sequencing. Sequences for both strands were edited and a consensus sequence was produced using Chromas, version 2.31 (Technelysium Pty Ltd.).

The stability of sequence mixed base heights was investigated by analyzing 10 separate PCR - ITS amplification and subsequent sequencing using genomic DNA of Kumo (14518) and 10 using genomic DNA of hybrid 14520. These analyses were conducted as per the other genomic DNAs above.

RESULTS AND DISCUSSION

Sequencing of nrDNA of *C. japonica* cv. *Haara* resulted in 1179 bp that agreed well with AB23983 in GenBank. However, sequencing of cv. *Kumotooshi* (Kumo) proved difficult and only 802 bp was cleanly sequenced. Nevertheless, the Kumo sequence was identical to that of Haara, except in the positions 154, 468, and 505. In Kumo, each of these three positions was heterozygous, which seems to imply that Kumo, itself, may be of hybrid origin. Of course, these could be relictual from incomplete lineage sorting. In any case, we do know that parent Kumo did contain (from some event) heterozygous bases at three positions in its nrDNA. Thus, although not ideal, it is still instructive to follow the fate of these three positions in the artificial hybrids. Three of the hybrids had nrDNA very similar to Kumo, with some variation in the ratio of bases. Unfortunately, repeated sequencing from both forward and reverse was unable to obtain clean sequences in the area around site 505 for six of the hybrids (NA in Table 1). The nrDNAs of four hybrids (Table 1) were the same as Haara at positions 154 and 468.

Table 1. Variable sites in the nrDNA sequence for *Cryptomeria japonica* cv. *Haara, C. japonica* cv. *Kumotooshi* and their hybrids. The ratios of bases in parenthesis ( ) were obtained by measurements of the peak sizes on the chromatogram. NA = not available.

					petN-psbM (from pollen)		
	site 154	site 468	site 505	nrDNA type	site 145	site 146	
Haara	C	T	G	Haara	A	T	Haara
Kumo	S(C/G 1:0.71)	Y(C/T 1:0.64	R(A/G 1:0.76)	Kumo	C	G	Kumo
Hybrids:							Pollen parent
14519	S(C/G 1:0.7)	Y(C/T 1:1)	R(A/G 1:0.7)	Kumo	C	G	Kumo
14520	S(C/G 1:0.71)	Y(C/T 1:0.46)	R(A/G 1:0.58	Kumo	C	G	Kumo
14521	S(C/G 1:0.2)	Y(C/T 0.5:1)	NA	Kumo	C	G	Kumo
14522	C	T	NA	Haara	C	G	Kumo
14523	C	T	NA	Haara	C	G	Kumo
14524	C	T	NA	Haara	C	G	Kumo
14525	C	T	NA	Haara	C	G	Kumo

Sequencing of petN-psbM yielded two SNPs at sites 145 and 146 (Table 1), revealing that all the hybrids had Kumo as the male, pollen parent and Haara as the maternal (seed) parent. The inheritance of nrDNA patterns appears un-correlated with the pollen source (Table 1).

The stability of sequence base heights was investigated by analyses based on 10 separate PCR - ITS amplification and subsequent sequencing for Kumo (14518) and 10 for hybrid 14520. The results are very uniform (Table 2). Although, the first PCR cycle might preferentially amplify a given DNA strand, there seems no support from these data that this is happening for these two genomic DNAs.

Table 2. Variation in base height on chromatograms at positions 154, 469 and 505 for ITS sequences.

rep.	154, C/G	469, C/T	505, A/G
14518-1	1:0.7	1:0.55	1:0.7
14518-2	1:0.7	1:0.6	1:0.7
14518-3	1:0.7	1:0.6	1:0.9
14518-4	1:0.7	1:0.7	1:0.8
14518-5	1:0.7	1:0.6	1:0.8
14518-6	1:0.7	1:0.7	1:0.8
14518-7	1:0.7	1:0.6	1:0.8
14518-8	1:0.8	1:0.7	1:0.7
14518-9	1:0.7	1:0.7	1:0.7
14518-10	1:0.7	1:0.6	1:0.7
avg +/- 2 SD	1:0.71+/-0.062	1:0.635+/-0.116	1:0.76+/-0.14
14520-1	1:0.8	1:0.7	NA
14520-2	1:1.0	1:0.4	1:0.5
14520-3	1:1.0	1:0.5	1:0.7
14520-4	1:1.0	1:0.4	1:0.6
14520-5	1:1.0	1:0.4	1:0.6
14520-6	1:1.0	1:0.5	1:0.5
14520-7	1:1.0	1:0.4	1:0.6
14520-8	1:0.9	1:0.4	1:0.6
14520-9	1:0.8	1:0.4	1:0.5
14520-10	1:1.0	1:0.5	1:0.6
avg +/- 2 SD	1:0.95+/-0.17	1:0.46+/-0.194	1:0.58+/-0.134

CONCLUSION

Although it was unfortunate that one of the parents (Kumo) had heterozygous nrDNA, the results showing that three of the seven (3/7) hybrids had nrDNA that was heterozygous, just as found in Kumo, whereas four of the seven (4/7) hybrids had nrDNA that was exactly like that of one parent, Haara. If these results can replicated, this appears to raise a cautionary flag on the use of nrDNA in detecting hybridization and favoring a more wide-spread use of single copy nuclear (SCN) genes for the analysis of putative hybridization.

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Ficus microcarpa (Moraceae) naturalized in Southern California, U. S. A.: Linking plant, pollinator, and suitable microhabitats to document the invasion process

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ABSTRACT

Ficus microcarpa is native to temperate and tropical Asia, Australasia, and Pacific regions. It is a popular ornamental tree grown in many warm temperate, subtropical, and tropical regions of the world, where it is widely known to escape from cultivation. It is reported here as being naturalized in Los Angeles, Orange, Riverside, San Diego, and Ventura counties, southern California. The invasive spread of *F. microcarpa* follows the introduction of its host-specific pollinating wasp, *Eupristina verticillata*; *E. verticillata* was first reported for California in 1994 from Arcadia, Los Angeles County. The wasp introduction reunited the *F. microcarpa* host plant–*E. verticillata* obligate pollinator mutualism thereby enabling the reproduction and naturalization of both organisms in California. A map showing the current distribution of *F. microcarpa*, citation of voucher specimens, and photographic documentation are provided. Published on-line www.phytologia.org *Phytologia 98(1):42-75 (Jan 5, 2016).* ISSN 030319430.

KEY WORDS: *Ficus microcarpa*, biological invasions, California, epiphytes, *Eupristina verticillata*, fig wasps, halophytes, invasive plants, mutualisms, ornamental horticulture, urban weeds.

Ficus (Moraceae) is one of the largest and most diverse genera of flowering plants. *Ficus* species occur primarily in subtropical and tropical regions around the world (Berg & Corner 2005). Members of the genus are treated within six subgenera based primarily on their differences in habit and inflorescence morphologies (Berg 2003), and species-specific wasp-dependent pollination syndromes (van Noort & Rasplus 2015). *Ficus* inflorescences are small and borne on the inner walls of a fruit-like and fleshy urn-shaped receptacle, i.e., the syconium, commonly known as a fig (Janzen 1979; Wunderlin 1997). Corner (1997), in a creative portrayal, described the syconium as a "cluster of flowers within a vase." Figs (syconia) are unique enclosed inflorescences that support complex coevolved mutualisms of pollinating and non-pollinating wasp communities (Janzen 1979; Wang et al. 2015). Fig pollination is often regarded as a model for study of coevolution, population genetics, host-parasitoid interactions, community ecology, historical biogeography, and conservation biology (Wieblen 2002).

Ficus microcarpa L.f. (subgenus *Urostigma*, section *Conosycea*) is an evergreen, monoecious tree native from Sri Lanka through India to southern China, Singapore, Taiwan, Japan, the Ryukyu Islands, northern Australia, New Caledonia, and many Pacific Islands, where it grows from sea level to about 1,800 m elevation (Wagner et al. 1999; Berg & Corner 2005; Tan et al. 2009; van Noort & Rasplus 2015; USDA GRIN 2015). *Ficus microcarpa* is a widely planted and popular ornamental tree, even within its native range, that has been introduced to many tropical, subtropical, and warm temperate regions around the world (Dehgan 1998; Rauch & Weissich 2000; Burrows & Burrows 2003; van Noort & Rasplus 2015).

In California, *F. microcarpa* is frequently cultivated along streets in coastal regions because it tolerates salt, wind, drought, and various types of soils, and it is often used for hedges (Brenzel 2007;

Hatch 2007; Perry 2010). *Ficus microcarpa* is extremely hardy, but is sensitive to frost, which restricts its use as an outdoor ornamental (Brenzel 2007; Tan et al. 2009; Perry 2010).

Throughout its range, *F. microcarpa* is known by many common names, including Chinese banyan, Indian laurel, laurel fig, curtain fig, Malay banyan, small-fruited fig, glossy-leaved fig, and many others (Tan et al. 2009; USDA, GRIN 2015; USDA, NRCS 2015). With a long history of use in the horticultural industry and numerous cultivars (Doty & Johnson 1954; Krishen 2006), there has been considerable nomenclatural confusion. The most frequently misapplied names for North American references include *F. nitida* sensu auct. non Thunb., *F. nitida* sensu auct. non L., and *F. retusa* sensu auct. non L. The APC (2015), The Plant List (2015), and Porcher (2015) provide a full synonymy. Confusion in the taxonomic literature began when Ridley (1924) mistakenly referred the Malay banyan to *Ficus retusa* L. (Tan et al. 2009). *Ficus retusa* L. and *F. microcarpa* L.f. are distinct species (Berg 2004; Berg & Corner 2005; The Plant List 2015; van Noort & Rasplus 2015). This misunderstanding persists, particularly among horticulturalists, and many nurseries continue to retail *F. microcarpa* under the old names.

Outside of cultivation, *F. microcarpa* grows on rocks, cliffs and hills, particularly on limestone, along rocky coasts, in beach forests, on floodplains and banks of tidal rivers, along the edge of swamps and mangroves, in rain forests, and frequently as an epiphyte on other trees (Chew 1989; Keng et al. 1990; Corner 1997; Weber 2003; Berg & Corner 2005). In coastal habitats it is often exposed to salt spray and is tolerant of water-logged soils within a wide range of salinity concentrations (Corner 1997; Tan et al. 2009; Yeo & Tan 2011). Accordingly, *F. microcarpa* has been identified as a halophyte (Menzel & Lieth 2003; Yensen 2015). Further, Aronson (1989) characterized it as a hydro-halophyte. In Malaysia, *F. microcarpa* can form monocultures at coastal sites even within its native range (Berg & Corner 2005). *Ficus microcarpa* is also renowned for its adaptability and tolerance to dry, harsh conditions in its native range and where it has been introduced. Throughout its range, it grows in tropical dry forests or dry coastal sites, fragmented and disturbed habitats, summer-dry Mediterranean climates, and frequently in urban environments (Starr et al. 2003; Weber 2003; Anbarashan & Parthasarathy 2013; Uotila 2015).

Ficus species are ecologically significant, keystone organisms because many animals feed upon their figs (Basset et al. 1997; Harrison 2005; Chaudhary et al. 2012). The syconia of *F. microcarpa* are axillary, grow singly or in pairs, measure about 6–10 (12) mm in diameter, and turn pinkish to reddish-green, purplish or black at maturity (Wagner et al. 1999; Berg & Corner 2005; Tan et al. 2009). Its figs are consumed by more than 200 frugivorous vertebrate species, primarily birds, but also bats, rodents, other small mammals, and ants, which act as secondary dispersal agents (Kaufmann et al. 1991; Shanahan et al. 2001; Starr et al. 2003). Pollinated figs of *F. microcarpa* contain small seeds that are easily dispersed and propagate readily in the boughs of host trees, in rock crevices, or on structures like buildings and bridges (McKey 1989; ISSG 2015).

Like most species of the subgenus *Urostigma*, *F. microcarpa* is a strangler fig. The subgenus *Urostigma* consists of about 280 species worldwide and most of them are hemi-epiphytes; i.e., the banyans and stranglers (Rønsted et al. 2008). Banyans produce aerial roots that later become accessory trunks, and the stranglers, which form root baskets around trunks of host trees, begin life as an epiphyte. The strangler fig seeds often germinate about 20–25 m above the forest floor, avoiding competition with ground-dwelling species, thereby increasing survivorship in dense rainforests where little light reaches the ground (Laman 1995; Berg & Corner 2005). Hemi-epiphytes produce aerial, adventitious, or creeping roots that grow down to the ground, which is followed by a transition to an independent or nearly free-standing tree that envelope or gradually kills its host (Berg & Corner 2005). The hemi-epiphyte *Ficus* species utilize host trees for support, but as a result of shading and competition for sunlight, limb breakage, mechanical restriction of trunk growth, and vascular constriction of phloem and xylem tissues caused by the *Ficus*, the host tree often

perishes (Putz & Holbrook 1985; Todzia 1986; Kramer 2011).

Many *Ficus* species that are hemi-epiphytes are also lithophytes (Berg & Corner 2005). *Ficus* lithophytes naturally occupy sunny cliff and rock habitats, and in urban environments they readily colonize open niches on buildings and other structures (Jim 1998). In fact, Shuyi (2009) found that many weedy *Ficus* species, which mostly belong to the subgenus *Urostigma*, are hemi-epiphytes and/or lithophytes that frequently inhabit anthropogenic and/or open natural cliff and rock habitats.

Species of *Ficus* exhibit one of the most extreme obligate nursery mutualisms known in nature and represent one of the few cases where active pollination behavior by fig wasps (Family Agaonidae; Hymenoptera) has evolved (Janzen 1979; Herre et al. 1996; Cook & Rasplus 2003; Yang et al. 2013). These wasps possess extreme host specificity and life cycles that are closely synchronized with the host fig's phenology (Wieblen 2002; Cruaud et al. 2010). *Ficus* species, therefore, can only be pollinated by their host-specific agaonid wasp, and in turn, the agaonid wasp can only reproduce if its associated species-specific *Ficus* syconia are present (Janzen 1979; Weiblen 2002; Starr et al. 2003); with some exceptions (Machado et al. 2005). Accordingly, non-native species of *Ficus* that establish in new regions do not produce fertile seed until their species-specific pollinators have also been introduced (Ramirez & Montero 1988).

The Old World fig wasp *Eupristina verticillata* Waterston (Agaonidae) is the pollinator of *F. microcarpa* (Ramirez & Montero 1988; van Noort & Rasplus 2015). Sexual reproduction is possible only if this specific fig wasp pollinator is present (Wang et al. 2015). There are a number of examples that document the transportation between countries and/or continents, and the re-establishment of the *F. microcarpa* host plant–*E. verticillata* obligate pollinator partnership that results in mutual reproduction of the two organisms. Prior to the 1920s–1930s, about the time when *E. verticillata* was purposely introduced to Hawai'i for reforestation, *F. microcarpa* produced neither mature fruit nor fertile seed in Hawai'i, and did not spread beyond cultivation (Pemberton 1939; Stange & Knight 1987; Beardsley 1998). In Florida, *F. microcarpa* was widely planted as an ornamental and a street tree, but began to spread outside cultivation only after *E. verticillata* arrived accidentally, sometime in the 1970s (Kaufman & Kaufman 2012). Apparently, the wasp was an unintentional introduction that may have been transported live in fruit from other infestations (McKey 1989). More recently, *E. verticillata* was unintentionally introduced to southern California where it was discovered in 1994 in Arcadia, Los Angeles County (CDFA 1994; Beardsley 1998). The J.W. Beardsley voucher specimen is housed at the Bernice Pauahi Bishop Museum in Honolulu (BPBM); California: Arcadia, ca. 600 ft., viii.1994, reared ex. fruit of *Ficus microcarpa* L.f. During the last 50 years, *E. verticillata* has also been accidentally or intentionally introduced and established in several other Mediterranean climate and desert regions, including Italy, Madeira, Malta, Canary Islands, Spain, Tunisia, Turkey, and the United Arab Emirates; see Wang et al. (2015) for a global review.

In the continental United States and its territories, *F. microcarpa* is reported growing outside of cultivation in Florida, Hawai'i, and Puerto Rico (Wunderlin 1997; USDA, NRCS 2015). Wunderlin (1997) noted that *F. microcarpa* was observed growing outside of cultivation in the Los Angeles area, southern California, but voucher specimens were not available for examination and it was not included in his treatment. *Ficus microcarpa* was first reported for California by O'Brien (1995) from the Los Angeles Civic Center area, with a second unconfirmed report from Pacific Palisades, also in Los Angles County. Randall (1997) in a CalEPPC Weed Alert cited O'Brien and reported *F. microcarpa* as possibly established or persistent. CNPS (2007) informally noted it growing at Echo Park and escaping elsewhere in downtown Los Angeles. The Consortium of California Herbaria (CCH 2015), however, posts records of ornamental trees persistent in old neighborhoods near El Segundo and on the Palos Verdes Peninsula, Los Angeles County, but no records for naturalized populations in Los Angeles County or elsewhere. Apparently, voucher specimens were never collected and distributed to herbaria to document early observations of the species escaping cultivation.

Ficus microcarpa, therefore, has not been cited for California by The Jepson Manual, Second Edition, or online by The Jepson Flora Project (Whittemore & McClintock 2012; Jepson Flora Project 2015). It has also not been included in other California publications that identify non-native species growing spontaneously outside of cultivation, including Hrusa et al. (2002), DiTomaso and Healy (2003, 2007), Roberts et al. (2004), Rebman and Simpson (2006), Clarke et al. (2007), Grewell et al. (2007), Dean et al. (2008), Roberts (2008), and Prigge and Gibson (2012).

Following the introduction of its host-specific pollinating wasp (*E. verticillata*), *F. microcarpa* has naturalized and is reported here growing spontaneously outside of cultivation in Los Angeles, Orange, Riverside, San Diego, and Ventura counties, southern California, U.S.A. *Ficus microcarpa* is widespread in urban environments where it grows on old masonry and concrete structures such as bridges, freeway underpasses, drainage channels, gutters, and sidewalk curbs. It is also epiphytic on the trunks of landscape trees, primarily palm trees. *Ficus microcarpa* also grows on the bank of an urban tidal channel and along the seashore on a harbor bulkhead within the salt spray zone. In native plant communities, *F. microcarpa* grows rarely in ephemeral calcareous-saline seeps in crevices of cliff and rock outcrops along the immediate coast.

For urban and native plant community sites, the plant appears to germinate from seed dispersed in figs eaten by birds or small mammals, and the seedlings establish and grow without human intervention or intentional summer watering. *Ficus microcarpa* is to be expected elsewhere in similar habitats at other localities wherever conditions for its growth are favorable and wherever its pollinating wasp co-occurs with ornamental plantings or expanding naturalized populations. Discussions about its naturalized habitats, substrate ecology, urban weeds, adaptive field survey protocols, and invasive plant status are provided. A distribution map, citation of voucher specimens, and photographic documentation of occurrences in southern California are also included.

MATERIALS AND METHODS

In October 2008, an approximately 1 m tall shrub was discovered growing in the crevice of a coastal rock outcrop seep in Laguna Beach, southern California. In July 2013, an approximately 1.2 m tall shrub was discovered growing in the crevice of a concrete-grouted stone wall along the seashore, also in Laguna Beach. These small shrubs were tentatively identified as *F. microcarpa*. Therefore, casual surveys were implemented to collect material with syconia and confirm the identification of *F. microcarpa* spreading outside of cultivation in southern California.

In February 2014, a nearly 3 m tall naturalized tree with syconia was discovered at Dana Point in Orange County, and in July, 2014, an approximately 3.5 m tall tree with syconia was documented in San Pedro, Los Angeles County. These two trees enabled confirmation of species identification.

Focused Field Survey Program

Following collection of fertile plants and a review of the literature, a focused survey program was designed and implemented in 2014-2015 to identify the naturalized distribution and determine the invasive status of *F. microcarpa* for southern California. Urban habitats including old masonry, concrete structures, and the trunks of palms and other landscape trees, particularly localities in close proximity to cultivated *F. microcarpa* trees, were the focus of surveys conducted for this study. Specific localities were selected for study, including the City of Arcadia, where its pollinator, *E. verticillata*, was first discovered. Other parts of the Los Angeles metropolitan area, including Echo Park and Pacific Palisades were also visited to verify early reports of juvenile *F. microcarpa* plants. Particular attention was devoted to surveying tidal wetlands and calcareous cliff habitats in native communities during routine travels across coastal southern California.

Wherever *F. microcarpa* was located outside of cultivation, data collection included a physical description of the location, GPS coordinates, notes on plant size, substrate, presence of syconia, and photographic documentation. Herbarium specimens were collected to voucher each population, with the exception of eight sites where plants were observed out of reach on upper palm tree trunks; those sites were photographed for documentation. In addition, soil samples were collected from urban masonry, cliff and rock outcrops, and tidal channel habitats and sent to Wallace Laboratories (El Segundo, California) to determine pH, salinity, and calcium carbonate (lime) content to establish preliminary substrate affinities. Citation of voucher specimens follows.

Vouchers: **U.S.A.: CALIFORNIA: Los Angeles Co.:** City of Los Angeles, Boyle Heights, S of Interstate 10, E side of Interstate 5 on bridge at State St., 34°03'11.5848"N 118°12'48.7050"W, elev. ca. 107 m, 1.2 m tall sapling growing in concrete bridge expansion joint, 23 Nov 2013, *Riefner 13-356* (RSA); City of San Pedro, W side of South Harbor Blvd. at West Santa Cruz St., 33°44'39.9156"N 118°16'47.1114"W, elev. ca. 6 m, 0.8 m tall sapling on palm trunk, 13 Jul 2014, *Riefner 14-243* (CDA, RSA); City of San Pedro, Port of Los Angeles, near Los Angeles Maritime Museum, Sampson Way at West 6th St., 33°44'17.7468"N 118°16' 43.3624"W, elev. ca. 1 m, ca. 3.5 m tall tree w/syconia, growing on bulkhead over breakwater rocks within the salt spray zone, 13 Jul 2014, *Riefner 14-245* (RSA, UC, UCR); City of Long Beach, N of East 2nd St. and W of Marine Stadium Park, Alamitos Bay, near docks on Bay Shore Ave. between Monroe Ave. and East Vista St., 33°45' 37.0260"N 118°07' 30.2406"W, elev. ca. 6 m, several 1-1.5 m tall multi-branched saplings on palm tree trunk, growing with *Nephrolepis cordifolia*, 27 Jul 2014, *Riefner 14-260* (CDA, RSA, UCR); City of Long Beach, Belmont Shores, N side of Ocean Blvd. at Prospect Ave., 33°45' 28.8144"N 118°08' 33.6084"W, elev. ca. 4 m, 2 m tall tree w/syconia on palm trunk, 11 Aug 2014, *Riefner 14-284* (CDA, RSA); City of Long Beach, Belmont Shores, N side of Ocean Blvd. between Quincy Ave. and Park Ave., 33°45' 26.8164"N 118°08'28.4510"W, elev. ca. 4 m, several 3- 8 cm tall seedlings growing in crevices along sidewalks and in gutters and several 1-1.5 m tall saplings on palm trunks, 11 Aug 2014, *Riefner 14-287 & 14-288* (CDA, RSA, UCR); City of Long Beach, Belmont Shores, N and S sides of Ocean Blvd. at Termino Ave., 33°47'17.2320"N 118°16' 54.8944"W, elev. ca. 11 m, 5 cm tall seedling growing in street gutter and sapling < 1 m tall on a palm trunk, 11 Aug 2014, *Riefner 14-292* (CAS); Harbor City, W of 110 Freeway, on Figueroa Pl. ca. 0.3 mi N of Anaheim St., opposite of Harbor Park Golf Course, 33°47'17.2320"N 118°16'54.8944"W, elev. ca. 9 m, two trees w/syconia ca. 4.5 - 5.2 m tall and intermingled saplings growing in joints of rough-grouted concrete wall, 6 Oct 2014, *Riefner 14-369* (CAS, RSA, UCR); Harbor City, W of 110-Freeway, Figueroa Pl. at Lagoon Dr., 33°46'54.5736"N 118°16'51.3297"W, elev. ca. 6 m, 3.5 m tall tree w/syconia growing in joint of rough-grouted concrete wall, 6 Oct 2014, *Riefner 14-371* (RSA, UCR); Harbor City, underpass on West L St. at Figueroa Pl., W side of 110 Freeway, 33°47'09.3876"N 118°16'53.7267"W, elev. ca. 6 m, ca. 1.5 m sprawling tree growing in joint of concrete wall ca. 8 m above ground, 6 Oct 2014, *Riefner 14-373* (RSA, UCR); City of Los Angeles, Westlake, Echo Park, S side of Bellevue Ave. near Glendale Blvd., 34°04'12.9828"N 118°15'39.0865"W, elev. ca. 121 m, 2 m tall saplings growing on upper palm trunk and crown foliage, 18 Apr 2015, *Riefner 15-112 photographic documentation*; City of Los Angeles, Westlake, Echo Park, Echo Park Ave., N of Bellevue Ave. and S of Laguna Ave., 34°04'18.7572"N 118°15'36.2852"W, elev. ca. 122 m, 1 - 2 m tall saplings growing on upper palm trunk and crown foliage, 18 Apr 2015, *Riefner 15-113 photographic documentation*; City of Los Angeles, Westlake, E side of Rampart Blvd. immediately S of West 3rd St., 34°04'02.9892"N 118°16'41.3940"W, elev. ca. 95 m, 1.5 m tall saplings growing on palm trunk among crown foliage, 18 Apr 2015, *Riefner 15-115 photographic documentation*; City of Los Angeles, Westlake, MacArthur Park, W of South Alvarado St. along 7th St., 34°03'24.6708"N 118°16'39.1415"W, elev. ca. 84 m, 1.8 m tall stout saplings growing on palm trunks and crown foliage, 18 Apr 2015, *Riefner 15-118* (RSA, UCR); City of Los Angeles, Lincoln Heights, S side of West Avenue 26 at Interstate 10 bridge, 34°04'59.8512"N 118°13'18.6930"W, elev. ca. 109 m, 1 m tall sapling and 2.2 m tall tree growing in joints of a concrete bridge and freeway retaining wall, 25 Apr 2015, *Riefner 15-127* (RSA); City of Los Angeles, Mt. Washington, W side of North Figueroa St. at Woodside Dr., 34°05'53.4120"N 118°12'19.9891"W, elev. ca. 140 m, 1.2 m tall stout sapling growing in crevice of stone wall, 25 Apr 2015, *Riefner 15-129* (RSA, UCR); City of Santa

Monica, both sides of Ocean Ave., N and S of Santa Monica Blvd., 34°00'47.9880"N 118°29'50.9528"W, elev. ca. 30 m, several 0.5 - 1.2 m tall saplings growing on the trunk and crown of palm trees, 25 Apr 2015, *Riefner 15-132* (RSA); City of Santa Monica, Pacific Palisades, along Sunset Blvd. at Pampas Ricas Blvd./Chautanqua Blvd. intersection, 34°02'27.8268"N 118°31'06.8789"W, elev. ca. 101 m, 1.5 m tall stout sapling growing in the crown of a palm tree, 25 Apr 2015, *Riefner 15-134 photographic documentation*; City of Arcadia. S side of Interstate 210, W of Colorado Blvd. between Second St. and La Porte St., 34°08'42.1728"N 118°01'33.2106"W (approximate data), elev. ca. 153 m, 2.5 m tall tree growing beneath freeway overpass in curb crevice along paved parking lot, 2 May 2015, *Riefner 15-138* (CDA, RSA); City of Glendale, Los Angeles River, Glendale Narrows Riverwalk, Flower St. W of Fairmont Ave., 34°09'23.7960"N 118°17'00.7107"W, elev. ca. 146 m, 1 m tall shrub growing in joint of a concrete retaining wall along river channel, 2 May 2015, *Riefner 15-141* (CDA, RSA); City of Glendale, Los Angeles River, Glendale Narrows Riverwalk, Fairmont Ave. E of Flower St., 34°09'23.1912"N 118°16'53.1647"W, elev. ca. 145 m, 1 m tall shrub growing in joint of concrete retaining wall on river channel at culvert outflow pipe, 2 May 2015, *Riefner 15-143 photographic documentation*; City of Carson, E side of 110 Freeway along 190th St., 33°51'41.9220"N 118°17'04.7618"W, elev. ca. 11 m, two ca. 2 m shrubs growing on the underpass wall and one 2 m tall shrub w/syconia growing in sidewalk/curb crack, E side of the Dominquez Channel, 19 Jul 2015, *Riefner 15-200* (CAS, CDA, RSA, UCR); City of Carson, Dominguez Channel at Figueroa St., S channel bank, 33°51'28.9836"N 118°16'53.7498"W, elev. ca. 7 m, single 2.2 m tall shrub w/syconia growing on channel bank with *Parietaria judaica* adjacent to tidal waters with *Atriplex* and *Salicornia*, 19 Jul 2015, *Riefner 15-203* (CAS, RSA); City of Los Angeles, on 43rd St. bridge, W side of 110 Freeway, E of Flower St., 34°00'19.6992"N 118°16'53.4046"W, elev. ca., 58 m, single ca. 3.2 m tall tree w/syconia growing in concrete expansion joint, 26 Jul 2015, *Riefner 15-207* (CAS, CDA, RSA); City of Los Angeles, E side of Figueroa St. at 45th St., 34°00'09.5940"N 118°16'57.3457"W, elev. ca. 42 m, seedling < 30 cm tall at base of palm tree trunk, 26 Jul 2015, *Riefner 15-209* (CAS, RSA); City of Glendale, E side of Glendale Freeway (Hwy. 2), Ripple St. at Rosanna St., 34°06'18.5544"N 118°14'57.7060"W, elev. ca. 116 m, two 2 m tall shrubs w/syconia growing in concrete expansion joints on bridge overpass, 26 Jul 2015, *Riefner 15-211* (CAS, RSA, UC); City of Alhambra, N side of 110 Freeway on E side of Garfield St., 34°04'19.6536"N 118°07'21.8492"W, elev. ca. 129 m, single 1.5 m tall shrub growing in concrete expansion joint of bridge overpass, 2 Aug 2015, *Riefner 15-214* (CAS, RSA, UCR); City of Los Angeles, E side of North Mission Rd. between 101 Freeway and Caesar E. Chavez Ave., 34°03'13.8924"N 118°13'34.2949"W, elev. ca. 101 m, 2 Aug 2015, *Riefner 15-216* (CAS, CDA, RSA); City of Malibu, N side of Pacific Coast Highway, E ca. 0.25 mi from Coastline Dr., 34°02'31.0056"N 118°34'07.8423"W, elev. ca. 15 m, single 1.4 m tall shrub growing in crevice of concrete retaining wall, 15 Aug 2015, *Riefner 15-222* (CAS, RSA); City of Santa Monica, Pacific Palisades, N side of Pacific Coast Highway at Sunset Blvd., 34°02'20.0328"N 118°33'14.3335"W, elev. ca. 12 m, sapling on upper palm tree trunk, 15 Aug 2015, *Riefner 15-224 photographic documentation*; City of Covina, N of 10 Freeway, E side of Grande Ave. at Walnut Creek, general vicinity of Fairway Ln., 34°04'30.0540"N 117°52'20.6194"W, elev. ca. 150 m, 1.3 m tall shrub and seedling growing in joints of concrete drainage channel, 21 Aug 2015, *Riefner 15-341* (CAS, RSA, UCR); City of Los Angeles, Universal City, W side of Barham Blvd. immediately S of DeWitt Dr., 34°07'53.4540"N 118°20'41.4011"W, elev. ca. 238 m, 8 dm tall sapling on palm trunk at base of tree, 23 Aug 2015, *Riefner 15-346* (CDA, RSA); City of Los Angeles, Toluca Terrace, E side of Cahuenga Blvd., S ca. 0.2 mi from Magnolia Blvd., 34°09'59.6232"N 118°21'41.9063"W, elev. ca. 188 m, 2 dm tall seedling at base of cinder block wall along sidewalk, 23 Aug 2015, *Riefner 15-348* (CAS, RSA).

Orange Co.: City of Laguna Beach, vicinity of West St. and Pacific Coast Hwy., 33°30'17.5176"N 117°44' 52.3291"W, elev. ca. 8 m, 1 m tall shrub in seep on coastal bluff outcrop, 22 Oct 2008, *Riefner 08-312* (CAS, RSA), same locality, 3 Jun 2014, *Riefner 14-149* (RSA); City of Laguna Beach, Heisler Park near Recreation Point, SW of Cliff Dr. at Myrtle St., 33°32' 38.1084"N 117°47'34.1096"W, elev. ca. 7 m, stout shrub 1.2 m tall growing in crevice of concrete-grouted stone wall, 11 Jul 2013, *Riefner 13-139* (CDA, RSA); City of Dana Point, W ca. 0.1 mi from Island Way along Dana Point Harbor Dr., cliff immediately S of Santa Clara Ave. and Amber Lantern St., 33°27'48.3192"N 117°42'05.4726"W, elev. ca. 11 m, ca. 3 m tall tree w/syconia growing in sandstone cliff crevice, 24 Feb 2014, *Riefner 14-66* (CAS, RSA, UC, UCR); City of

Newport Beach, N side of Pacific Coast Highway at Riverside Ave., 33°37'13.4472"N 117°55'26.8840"W, ca. 6 m, seedlings in gutter and joint of concrete street drain, 14 Jun 2014, *Riefner 14-169* (CAS, RSA); City of Laguna Beach, N side of North Coast Highway near intersection with Cliff Dr., 33°32'37.1580"N 117°47'15.1785"W, elev. ca. 10 m, saplings growing in crevices of concrete-grouted stone wall, 21 Jun 2014, *Riefner 14-186* (CAS, CDA, RSA); City of Irvine, S side of Red Hill Ave. bridge E of 405 Freeway northbound lane, 33°41'14.6004"N 117°51'59.0304"W, elev. ca. 31 m, 2 m tall tree growing in concrete bridge expansion joint, 6 Jul 2014, *Riefner 14-222* (CAS, RSA); City of Seal Beach, N side of Westminster Blvd. ca. 0.1 mi W of Road B, 33°45'35.0784"N 118°05'05.4978"W, elev. ca. 3 m, ca. 1.2 m tall sapling growing in joint of concrete drainage channel, 13 Jul 2014, *Riefner 14-241* (CDA, RSA, UCR); City of Laguna Beach, E side of South Coast Highway near Diamond St., 33°31'40.4076"N 117°46'12.2079"W, elev. ca. 25 m, two stout saplings in crevices of concrete-grouted brick wall, 8 Aug 2014, *Riefner 14-273* (CAS, CDA, RSA); City of Irvine, W of Irvine Blvd. and S of Pusan Way, former MCAS El Toro facility, 33°40'42.9456"N 117°42' 56.1746"W, elev. ca. 138 m, scattered seedlings and saplings on palm trunks, growing with *F. rubiginosa*, 26 Nov 2014, *Riefner 14-399* (CDA, RSA); City of San Clemente, San Clemente Municipal Golf Course, Ave. San Luis Rey at Ave. Santa Inez, 33°24'20.1672"N 117°35'43.8156"W, elev. ca. 51 m, 1.8 m tall sapling growing on trunk of cultivated pine tree, 5 May 2015, *Riefner 15-149* (CDA, RSA, UCR); City of San Clemente, Ave. Santa Inez at El Camino Real, 33°24'16.8948"N 117°35'48.8648"W, elev. ca. 44 m, sapling on upper palm trunk, 5 May 2015, *Riefner 15-150 photographic documentation*; City of Costa Mesa, Anton Blvd. on ramp to 405 Freeway, ca. 0.3 mi from intersection of Bristol St. at Sunflower Ave., 33°41'17.6532"N 117°52'26.7539"W, elev. ca. 11 m, 2.5 m tall shrub w/syconia growing in expansion joint of concrete bridge, 18 Jul 2015, *Riefner 15-198* (CAS, RSA, UCR); City of Laguna Beach, S side of Beach St. and E of Broadway Ave. (Hwy. 133), 33°32'38.8428"N 117°47'00.8467"W, elev. ca. 7 m, 15 cm seedling growing at the base of a cinder block wall, growing with *F. rubiginosa*, 27 Aug 2015, *Riefner 15-355* (CAS, RSA); City of Newport Beach, Corona del Mar, on Marguerite Ave. between Fifth Ave. and Fourth Ave., 33°35'57.9012"N 117°52'03.7748"W, elev. ca. 41 m, broad-based tree (ca. 28 cm wide at base), ca. 3 m tall w/syconia growing on palm tree trunk at base of tree, and seedlings and saplings widespread on upper palm tree trunks on the same street, 27 Aug 2015, *Riefner 15-359* (CAS, UC, UCR); City of Newport Beach, Corona del Mar, along Marguerite Ave. S of Fourth Ave., 33°35'55.5468"N 117°52'06.0792"W, elev. ca. 41 m, 1 m tall shrub growing on palm tree trunk at base of tree, 27 Aug 2015, *Riefner 15-360* (CAS, RSA); City of Newport Beach, Corona del Mar, along Marguerite Ave. near Seaview Ave., 33°35'42.8244"N 117°52'19.2982"W, elev. ca. 30 m, 8 cm seedling growing on palm tree trunk at base of tree, scattered saplings on upper palm trunks, growing with *F. rubiginosa*, on the same street, 27 Aug 2015, *Riefner 15-366* (CAS, RSA); City of Rancho Santa Margarita, Trabuco Marketplace, Rancho Santa Margarita Blvd. at Plano Trabuco, 33°38'53.6028"N 117°34'36.3579"W, elev. ca. 363 m, seedling on palm trunk at base of tree, shrubs on upper palm trunks, growing with *F. carica* and *F. rubiginosa*, 29 Aug 2015, *Riefner 15-368* (CAS, CDA, RSA); City of Lake Forest, N side of Interstate 5 Freeway on E side of Lake Forest Dr. bridge, 33°37'42.4848"N 117°43'12.3416"W, elev. ca. 144 m, 1 m tall sapling growing in concrete expansion joint, 29 Aug 2015, *Riefner 15-372* (RSA); City of Anaheim, E side of Lemon St. at East Adele St., 33°50'18.7152"N 117°55'00.8405"W, elev. ca. 48 m, seedlings on palm tree trunk at base of tree and sapling on upper palm trunk, 30 Aug 2015, *Riefner 15-374* (RSA); City of Anaheim, Pearson Park, near center of park, N of North Helena St. and West Cypress St., 33°50'16.7316"N 117°55'05.3326"W, elev. ca. 45 m, 1 m tall shrubs on old masonry around rain gutter, and seedling on trunk of *Schinus terebinthifolius* at base of tree, 30 Aug 2015, *Riefner 15-376 & Riefner 15-377* (RSA); City of Anaheim, Pearson Park, SE section of park, N of North Helena St. and West Cypress St., near intersection of North Lemon St. and West Cypress St., 33°50'15.2664"N 117°55'01.4651"W, seedlings on the trunk of palm trees, *Dracaena draco*, *Olea europaea*, and *Pinus halepensis*, and on calcareous memorial stone, 30 Aug 2015, elev. ca. 44 m, *Riefner 15-379*, *Riefner 15-380*, *Riefner 15-381* (CAS), *Riefner 15-382 & Riefner 15-383* (RSA).

Riverside Co.: City of Corona, Corona City Park, E side of Rimpau Ave. on N side of Sixth St., 33°52'29.2224"N 117°33'10.1202"W, elev. ca. 211 m, 0.5 m sapling on palm trunk, 8 Aug 2015, *Riefner 15-217* (CDA, UCR); City of Corona, S side of 91 Freeway ca. 120 ft W of Lincoln St., N side of Corona del Rey Apartments, 33°52'53.8176"N 117°34'59.9012"W, elev. ca. 210 m, 1.5 m tall shrub growing from

crevice of cinder block wall, 8 Aug 2015, *Riefner 15-220* (CAS, CDA, UC, UCR).

San Diego Co.: City of La Jolla, E side of La Jolla Blvd. near Playa Del Norte St., 32°49'47.7732"N 117°16'36.5555"W, elev. ca. 24 m, 1.5 m stout shrub on palm trunk, 8 Aug 2014, *Riefner 14-271* (CDA, RSA); City of Encinitas, E side of 3rd St. between West G St. and West F St., 33°02'33.5436"N 117°17'42.9849"W, elev. ca. 22 m, seedling on palm trunk, growing with *F. rubiginosa*, 12 Oct 2014, *Riefner 14-377* (RSA, UCR); City of Encinitas, near intersection of Sylvia St. and La Veta Ave., 33°03'04.4712"N 117°17'50.9157"W, elev. ca. 17 m, ca. 3 m tall tree on palm trunk, growing with *F. rubiginosa*, 12 Oct 2014, *Riefner 14-380* (CAS, CDA, RSA, UCR); City of Carlsbad, Harding St. at Camellia Pl., 33°09'18.9504"N 117°20'21.4246"W, elev. ca. 30 m, ca. 2.5 m tall tree w/syconia on upper palm trunk, 30 Apr 2015, *Riefner 15-135 photographic documentation*; City of Carlsbad, along Adams St. near Larkspur Ln., 33°09'17.0424"N 117°20'06.0912"W, elev. ca. 26 m, seedling and 1.5 m tall shrub on palm trunks, 3 May 2015, *Riefner 15-144* (CDA, RSA, UCR); City of Carlsbad, along Adams St. near Magnolia St., 33°09'19.9260"N 117°20'08.3799"W, elev. ca. 27 m, seedling and 0.5 m tall sapling on palm trunks, 3 May 2015, *Riefner 15-147* (CAS, RSA); N of Oceanside, Camp Pendleton, S side of 16th St. ca. 0.1 mi W of Vandegrift Blvd., 33°18'44.5752"N 117°18'43.5613"W, elev. ca. 101 m, 1 m tall shrub rooted in concrete storm drain, 17 May 2015, *Riefner 15-154* (CAS, RSA, UC); City of Oceanside, E side of Carmelo Dr. near Harbor Dr., N of Goodland Dr., 33°12'29.8908"N 117°23'17.4163"W, elev. ca. 23 m, shrub < 1 m tall w/syconia and seedling on base of palm tree trunks, 17 May 2015, *Riefner 15-157* (CDA, RSA, UCR); City of San Marcos, Las Posas Rd. on N side of Highway 78, 33°08'39.6240"N 117°11'28.2435"W, elev. ca. 177 m, shrub < 1 m tall, on concrete bridge in expansion joint, 29 Jun 2015, *Riefner 15-178* (RSA, UCR).

Ventura Co.: City of Oxnard, E side of Oxnard Blvd. (Hwy. 1), S ca. 0.2 mi from West Wooley Rd., 34°11'18.2364"N 119°10'31.9567"W, elev. ca. 9 m, 5 dm tall seedling on palm trunk at base of tree, 16 Aug 2015, *Riefner 15-229* (CAS, RSA).

RESULTS

This study documented 66 populations of *F. microcarpa* naturalized in Los Angeles, Orange, Riverside, San Diego, and Ventura counties, southern California, which are depicted in Fig. 1. Of the documented sites, *F. microcarpa* is reported for the first time for Orange, Riverside, San Diego, and Ventura counties.

In southern California, naturalized *F. microcarpa* plants observed during field surveys range in size from seedlings < 10 cm tall, and saplings, shrubs or small trees growing to over 5 m tall. The six largest trees all bearing syconia were documented from urban and native habitats, including localities in Los Angeles County at Harbor City (Fig. 2), San Pedro (Fig. 3), and the City of Los Angeles (Fig. 4), at Newport Beach (Fig. 5) and Dana Point in Orange County (Fig. 6), and in San Diego County at Encinitas (Fig. 7). Many of the naturalized shrubs and trees in southern California produce figs, and most fertile plants are generally > 2.5 m tall. The smallest fig-bearing plant was a < 1 m tall shrub documented in Oceanside, San Diego County (Fig. 8).

The distribution of the largest trees and shrubs observed during this study is depicted in Fig. 1. The frequency of occurrence suggests that *E. verticillata* was first introduced (likely unintentionally) to southern California in the greater Los Angeles metropolitan area, i.e., first collected in 1994 from Arcadia (Fig. 1). However, the initial introduction may have occurred at a more coastal site, perhaps a port-of-call or the Los Angeles International Airport. O'Brien (1995) speculated that fig wasps may have arrived in the Los Angeles area in 1992, but an earlier date of introduction, perhaps in the 1980s, cannot be ruled out; neither can multiple points or sources of introduction.

Naturalized *F. microcarpa* juvenile plants and small shrubs were found, often unexpectedly, at several inland urban localities, such as Rancho Santa Margarita in Orange County, and Corona in western Riverside County. Based on these observations and the distribution of populations depicted in Fig. 1, *F.*

microcarpa is in the process of a rapid range expansion in southern California.

Southern California Naturalized Populations

Ficus microcarpa thrives outside its natural range wherever its pollinating fig wasp, suitable climate, substrates, and seed-dispersing animals are present. It is often prolific in urban environments (Corlett 2006; Shuyi 2009). In coastal southern California, *F. microcarpa* has established easily in the joints of old masonry, stone and brick walls (Fig. 9), and on concrete structures such as bridges, retaining walls (see Figs. 2 & 4), freeway underpasses, drainage channels, gutters, curbs and sidewalks. It is also epiphytic on the trunks of landscape trees, particularly on palm tree trunks at coastal and inland sites (Fig. 10), primarily *Phoenix canariensis* Chabaud (Arecaceae), but also on *Washingtonia filifera* (André) de Bary, *W. robusta* H. Wendl., and *Butia capitata* (Mart.) Becc. (Arecaceae). *Ficus microcarpa* is also epiphytic on the trunks of *Dracaena draco* (L.) L. (Asparagaceae), *Olea europaea* L. (Oleaceae), *Pinus halepensis* Miller, *P. pinea* L. (Pinaceae), and *Schinus terebinthifolius* Raddi (Anacardiaceae); taxonomy follows Baldwin et al. (2012) or FNA (2015). It also grows on an old wood post, and a bulkhead along harbor shores within the salt spray zone (see Fig. 3). *Ficus microcarpa* has also been found on the bank of an urban tidal channel, Dominguez Channel (Fig. 11). In native plant communities in southern California it has been found rarely on calcareous-saline cliff (see Fig. 6) and rock outcrops (Fig. 12) along the immediate coast.

Based on a preliminary sampling, laboratory analyses using saturation extracts of soils taken within the root zone along tidal shoreline habitat and cliff/rock outcrop crevices indicate the substrates are slightly to moderately alkaline (7.02–8.31 pH), slightly to highly calcareous (calcium carbonate), and range from very slightly saline to strongly saline (2.70–>16 dS/m^{-1}). The urban masonry substrate of *F. microcarpa* is moderately alkaline (8.06 pH), highly calcareous, and non-saline (1.01 dS/m^{-1}), which are the expected parameters for cement/concrete substrates (Wikipedia 2015).

At the cited localities, *F. microcarpa* appears to germinate from seed dispersed in figs eaten by birds or small mammals, which establish and grow spontaneously without the aid of human intervention and without intentional summer watering. At some urban sites, however, aerosol drift from irrigation and runoff from hard surfaces may be influential. At numerous coastal and inland sites in southern California, *F. microcarpa* grows on the upper trunks of palm trees well beyond the possible influence of urban waters. For these populations (Fig. 13), mist and ocean fogs, and the inland extent of the summer marine layer may likely provide the moisture needed for seedling establishment and persistence of *F. microcarpa* hemi-epiphytes in arid southern California.

The Urban Epiphyte Flora

Metropolitan areas are particularly vulnerable to non-native species introductions, which can serve as entry pathways for invasions from the urban environment to native ecosystems (van Ham 2013). Often overlooked in southern California, documentation of ornamental plants escaping in urban habitats can provide important information to track the early dispersal and naturalization of these plants in natural areas. *Nephrolepis cordifolia* (L.) C. Presl (Nephrolepidaceae), which grows outside of cultivation on concrete walls, trunks of palm trees, and calcareous cliffs and bluffs in southern California, is an excellent example (Riefner & Smith 2015). Interestingly, Brusati et al. (2014) listed 186 species of ornamentals of greatest concern for introduction and/or invasiveness in California through the horticultural pathway, including *N. cordifolia*, but not *F. microcarpa*. In southern California, *F. microcarpa* co-occurs with *N. cordifolia* at several locations, including native habitats on calcareous cliffs, and it is also epiphytic on the trunks of palm trees in urban landscapes (Fig. 14).

In southern California, it is not unusual to find several species of *Ficus* epiphytic on the same palm tree or along the same street in urban areas, including *F. carica* L. and *F. rubiginosa* Desf. ex Vent. (see Fig.

7), and numerous other plant species that have not been inventoried. Brandes (2007) provides an extensive list of non-native epiphytes growing spontaneously on *P. canariensis* in Mediterranean climate regions. Birds presumably act as the primary vector of dispersal, but wind may also spread spores and other propagules tree-to-tree or from cultivated and naturalized sources. The dispersal of non-native epiphytes on palm trees across urban southern California increases the opportunity for potentially invasive species to naturalize in suitable native habitats. Accordingly, the urban epiphyte flora of southern California requires further study and documentation.

DISCUSSION

Many non-native plants that have naturalized in floras worldwide have been the product of deliberate introductions (Reichard & White 2001; Mack & Erneberg 2002). In addition, many invasive plants are often of horticultural origin (Bell et al. 2003). In fact, more than 60% of the most invasive plants in California were purposefully cultivated (Bossard et al. 2000). Numerous studies also indicate that many species of cultivated *Ficus* naturalize, and often become weedy or invasive (Ramírez & Montero 1988; Nadel et al. 1992; Gardner & Early 1996; Shuyi 2009).

Setting the Stage for Naturalization and the Invasion Process

Once the *F. microcarpa–E. verticillata* mutualism was reunited, three major factors discussed below paved the way for the *F. microcarpa* invasion. First a suitable climate and abundance of cultivated *F. microcarpa* trees in the Los Angeles Basin facilitated the establishment and spread of its host-specific pollinator, *E. verticillata*. Establishment of the pollinator enabled production of fertile fruits that could be dispersed facilitating establishment of *F. microcarpa* outside of cultivation without direct human help. An abundance of fruit-eating birds in the urban forest was available to facilitate seed dispersal. In addition to urban structures, an abundance of palm trees having persistent (marcescent) leaf bases that trap and accumulate organic matter and moisture were present to provide abundant microhabitat establishment sites.

Eupristina verticillata, Pollinator of *Ficus microcarpa*

For the obligate *F. microcarpa–E. verticillata* mutualism, fig wasp abundance is dependent upon the host tree resource (Yang et al. 2013). *Eupristina verticillata* was first discovered near Arcadia, Los Angeles County, California.

The urban forest of the greater Los Angeles metropolitan area provides abundant host tree resources to facilitate the establishment and dispersal of *E. verticillata* in southern California. In the City of Los Angeles, approximately six million trees have been inventoried for the metropolitan area, including 182,170 *F. microcarpa* trees, which comprise 3% of the urban forest; *Cupressus sempervirens* L. is the most frequently planted at 457,180 trees or 7.6% of the urban forest (Nowak et al. 2001). The City of Santa Monica supports approximately 33,800 public trees, of which *F. microcarpa* is the second most frequently planted at 3,088 trees that comprise 9.1% of the urban tree resources (CSM 2015); noteworthy since early unconfirmed sightings of juvenile plants at Pacific Palisades were reported by O'Brien (1995).

Yang et al. (2013) examined the population dynamics of *E. verticillata* and the phenology of a seasonal-fruited 29-tree population of *F. microcarpa* located in Taipei, Taiwan. Their results revealed three seasons of annual fig production correlated with temperature; spring crop, summer-fall crop, and winter trough (low point) seasons. The *E. verticillata* population size showed an increasing trend in spring, reached maximum abundance in summer, and then declined drastically in winter, which is consistent with the seasonal pattern of fig production. Despite the small number of local *E. verticillata* surviving on winter

syconia, the *E. verticillata* population for this 29-tree urban stand can increase quickly from nearly zero to over 40,000 wasps within a season when the small number of wasps overwintering on *F. microcarpa* syconia is combined with potentially immigrating wasps from other *Ficus* populations. Thus, this phenological seasonal pattern of fig production, coupled with the fast recovery rate of an *E. verticillata* pollinator population, may explain the worldwide adaptation and invasion of *F. microcarpa* (Yang et al. 2013).

Thereby, the warm-temperate Mediterranean climate of southern California coupled with abundant *F. microcarpa* cultivated host tree resources facilitated the initial establishment and apparently rapid spread of *E. verticillata* following its initial discovery in 1994. During this phase of dispersal, *E. verticillata* likely had few parasitoid fig wasp competitors resulting in an abundance of *F. microcarpa* fertile seed production; see Wang et al. (2015) for further discussion.

Ficus microcarpa, the Urban Environment Connection

In many native habitats numerous frugivores, particularly birds, disperse *Ficus* seeds that germinate in the crevices of trees or rock outcrops (Basset et al. 1997; Shanahan et al., 2001; Harrison 2005; Chaudhary et al. 2012), but a similar scenario plays out in urban environments. *Ficus* seedlings are often conspicuous on structures and the trunks of landscape trees in cities, largely because many urban-dwelling birds consume and disperse fig seeds (Weber 2003; Corlett 2006; Tan et al. 2009). In Florida, Caughlin et al. (2012) also found that fig-eating birds are common in urban areas, which result in high rates of seed dispersal and establishment of *F. microcarpa* juvenile plants in city landscapes. In Hong Kong, *F. microcarpa* comprises 50% of the trees growing spontaneously on stone walls (Jim 1998). Accordingly, *Ficus* species are keystone resources in many urban environments (Lok et al. 2013).

Ficus microcarpa is frequently cultivated, its seed readily dispersed by urban animal vectors, and it is a naturally-occurring lithophyte that favors lime-rich alkaline substrates (Shuyi 2009; Tan et al. 2010; Jim & Chen 2011). *Ficus microcarpa* is therefore a notorious invader of old masonry and grows easily on buildings, bridges, and many other urban structures within its native and introduced range (Jim & Chen 2011). It also tolerates disturbance, nutrient-poor microhabitats, pollution, drought, and is highly adaptable to urban environments (Wen et al. 2004; Li et al. 2005; Shuyi 2009).

In urban southern California, many birds utilize ornamental landscape trees for nesting or roosting (Garrett 1997, 1998; Crooks et al. 2004). As part of the urban scenario, these birds continually defecate fig seeds consumed from cultivated trees, and *F. microcarpa* juvenile plants often establish nearby on structures or the trunks of palm trees.

Palm Tree Susceptibility to Strangler Fig Invasions

Plant morphological features such as epidermal texture and roughness play an important role in epiphyte recruitment (Compton & Musgrave 1993; Male & Roberts 2005). Bark stability and chemistry, water-holding capacity, tree architecture, and other variables can influence epiphyte performance (Wagner et al. 2015).

For the monocotyledons, particularly the palm family (Arecaceae), the presence of marcescent leaf bases (withering but not falling off) or if leaf bases are senescent with the fronds are important for epiphyte establishment. Recruitment of strangler figs on palms in urban Queensland, Australia, is frequently associated, but not always, with leaf base retention; conversely, palm tree species that cleanly sheath (abscission) old leaf bases generally preclude hemi-epiphyte *Ficus* invasion (McPherson 1999). Marcescent leaf bases promote the accumulation of detritus, thereby providing important microhabitats for hemi-epiphyte *Ficus* seed retention, germination, and seedling establishment (Kramer 2011). The detritus trapped behind persistent leaf bases is often higher in organic matter, nitrogen, magnesium, and

potassium than soils located on the ground at the base of the palm tree (Putz & Holbrook 1989). In South Florida, Kramer (2011) concluded palm species with marcescent leaf bases, i.e., the *Sabal* palms and *P. canariensis* were the most susceptible to *Ficus* hemi-epiphyte invasions. In addition, Aguirre et al. (2009) found the abundance and species richness of epiphytes was higher on palms than on non-palm tree substrates, and that *Ficus* hemi-epiphytes are strongly biased toward palm tree hosts.

In Mediterranean ecosystems, Brandes (2007) found that *P. canariensis* provides a specialized substrate for epiphyte recruitment, including *F. carica* and *F. microcarpa*. *Phoenix canariensis* is a popular landscape tree in warm climates throughout the world, and it is extensively planted in southern California (McMinn & Maino 1981; McPherson et al. 2001; Zona 2008; Trent & Seymour 2010), which likely aided the establishment and dispersal of *F. microcarpa* in southern California.

In addition to *P. canariensis*, the fan palms (*W. filifera*, *W. robusta*) are extensively cultivated along streets and parks in California (McMinn & Maino 1981). They are prized for their ease of culture, fast growth, and handsome ornamental form, and both palms are frequently planted together in urban landscapes (Hodel 2014). *Washingtonia robusta*, in particular, is one of the most common ornamental trees cultivated in southern California (McPherson et al. 2001; Nowak et al. 2010; CSM 2015). Although *Washingtonia* species have persistent leaf bases, the dead fronds persist and form a 'skirt' (Simono 2012). In a study of tree ferns, Brownsey and Page (1986) found the retention of dead fronds form a fringing skirt that deters establishment of epiphytes and climbing vines. In southern California, however, routine maintenance activities trim dead fan palm skirts of urban landscape trees thereby exposing the leaf-base niche and rough bark textures that provide favorable microhabitats for *Ficus* hemi-epiphyte recruitment.

Despite the humid environment of subtropical and tropical rainforests, hemi-epiphytes can be subjected to extremes of moisture availability, including drought-like conditions associated with the epiphytic habitat (DeNiro et al. 1985; Putz & Holbrook 1989). Accordingly, strangler figs have evolved special morphological and physiological adaptations to deal with the resource limitations imposed by the epiphytic environment, including waxy leaves with sunken stomata and fleshy stem tubers that alleviate water stress (Schmidt & Tracey 2006).

Given the nature of *Ficus* seed dissemination by urban birds, high propagule pressure associated with widespread cultivation, and adaptations of the strangler figs to alleviate water stress, it is not surprising to find *F. microcapra* utilizing *P. canariensis* and other palm tree substrates as a stepping stone for dispersal across southern California's urban environment.

Invasive Plant Status

In the Global Compendium of Weeds, *F. microcarpa* is listed as a weed, sleeper weed, agricultural weed, noxious weed, introduced species, garden escape, environmental weed (invasive, or species that invades native ecosystems), naturalized or a cultivation escape (Randall 2002; HEAR 2015). *Ficus microcarpa* is reportedly potentially invasive, weedy, or of environmental concern where its specialist pollinating wasp has also been introduced (Randall 2012; HEAR 2015).

Ficus microcarpa has been documented as an invasive species in the New World for Bermuda, Florida, Hawai'i, and Central and South America (Ramirez & Montero 1988; McKey 1989; Nadel et al. 1992; Weber 2003; Caughlin et al. 2012; Wang 2014; GB 2015; HEAR 2015; ISSG 2015). It is classified as an environmental weed in Australia (HEAR 2015), and in New Zealand, although not yet naturalized, *F. microcarpa* is recognized as a potential problem weed (HEAR 2015). In the Mediterranean region, *F. microcarpa* is mostly a weed of urban habitats (Schicchi 1999; Brandes 2007; Verloove & Reyes-Betancort 2011; Caughlin et al. 2012). In Israel, however, it is invasive but not widespread (Dufour-Dror 2013; EPPO 2015).

Invasions of *F. microcarpa* in the United States and its territories are well documented, with the exception of California. In Hawai'i, most of the main islands are infested with *F. microcarpa*, including disturbed urban sites and natural areas in wet and dry forests (Starr et al. 2003). In Florida, it is listed as a 'Category I' invasive plant, defined as alien plants that alter native plant communities by displacing species, change community structures or ecological functions, or they hybridize with natives (FLEPPC 2015). Although *F. microcarpa* has received some attention as possibly established and a potentially invasive species in California, it has not been rated or evaluated by Cal-IPC (2015).

Ficus microcarpa can propagate spontaneously from seed on many surfaces. If it is not removed, *F. microcarpa* can cause structural damage to concrete and buildings, and as an epiphyte it will eventually strangle the host tree (Weber 2003; HEAR 2015; ISSG 2015). In addition, *F. microcarpa* is a fast-growing tree that can shade out native plant species and modify competitive regimes of natural communities (Gordon 1998; ISSG 2015).

Currently, it appears that *F. microcarpa* is limited primarily to urban areas. Owing to high propagule pressure associated with widespread cultivation and the consequent large fig crop production, *F. microcarpa* will likely continue to expand its naturalized range and further invade natural areas in southern California. Native habitats most vulnerable to invasions include estuaries, floodplains and banks of tidal creeks, riparian scrub and woodlands, sloughs, and coastal bluff seeps, particularly calcareous-saline substrates. *Phoenix canariensis*, its principal host tree and an acknowledged halophyte (Menzel & Lieth 2003; Yensen 2015), is a known invader of riparian and estuary habitats in southern California (Roberts 2008; Talley et al. 2012). *Ficus microcarpa* hemi-epiphyte invasions will likely follow.

CONCLUSIONS

Mutualisms often structure ecosystems and mediate complex ecosystem functions, but they also facilitate biological invasions (Traveset & Richardson 2014). Plant species escaping cultivation must negotiate multiple biotic and abiotic barriers in order to survive, colonize, reproduce, and disperse to new sites (Richardson et al. 2000). The global movement of organisms and ornamental horticulture has promoted invasions (Mack & Lonsdale 2001; Brusati et al. 2014), sometimes by reuniting obligate plant–pollinator partnerships in new regions and environments. The *F. microcarpa–E. verticillata* mutualism represents one of the best known case studies of plant and pollinator–mediated naturalization and invasion processes, which now has also been documented for southern California.

For scientists and resource managers alike, it is logical to assume that cultivated or accidentally introduced plants with specialized pollination syndromes are unlikely to set seed. Specialized pollination makes these plants unlikely candidates for early detection management programs; they rarely find their way onto predictive invasive plant lists. Early detection and assessment are important and fundamental management objectives when dealing with invasive plant species (Rejmánek 2000). Unfortunately, early reports of new species in local floras or journals often go unnoticed by natural area managers, and voucher specimens may not be submitted to herbaria for formal documentation. Accordingly, a potentially invasive non-native species may only be recognized as troublesome decades after it was first detected (Randall 1997). Such is the case here for *F. microcarpa*. Rejmánek (2000) remarked that we should pay more attention to habitat-specific predictors, which in this scenario, should include urban environments, and microhabitats such as palm tree trunks as substrates for epiphyte invasions.

Ficus microcarpa, widely cultivated in southern California, is highly adaptable to the summer-dry Mediterranean climate. Its pollinator too, *E. verticillata*, is highly adaptive to variable syconia production in seasonal climate regimes. Once this plant–pollinator mutualism was reunited, *F. microcarpa* seeds were readily dispersed by animal vectors and germinated in microhabitats suitable for a naturally-

occurring lithophyte that favors lime-rich alkaline substrates of urban structures, as well as the abundant micro-niches provided in the palm tree-rich urban landscape favored by these hemi-epiphytes. Accordingly, *F. microcarpa* is in the process of a rapid range expansion in southern California's urban environment, which will likely lead to expanded invasions of natural area habitats. Given the propensity of *Phoenix* and *Washingtonia* palms to invade estuarine and riparian ecosystems, land managers and scientists should carefully monitor these habitats for *F. microcarpa* invasive occurrences in coastal southern California.

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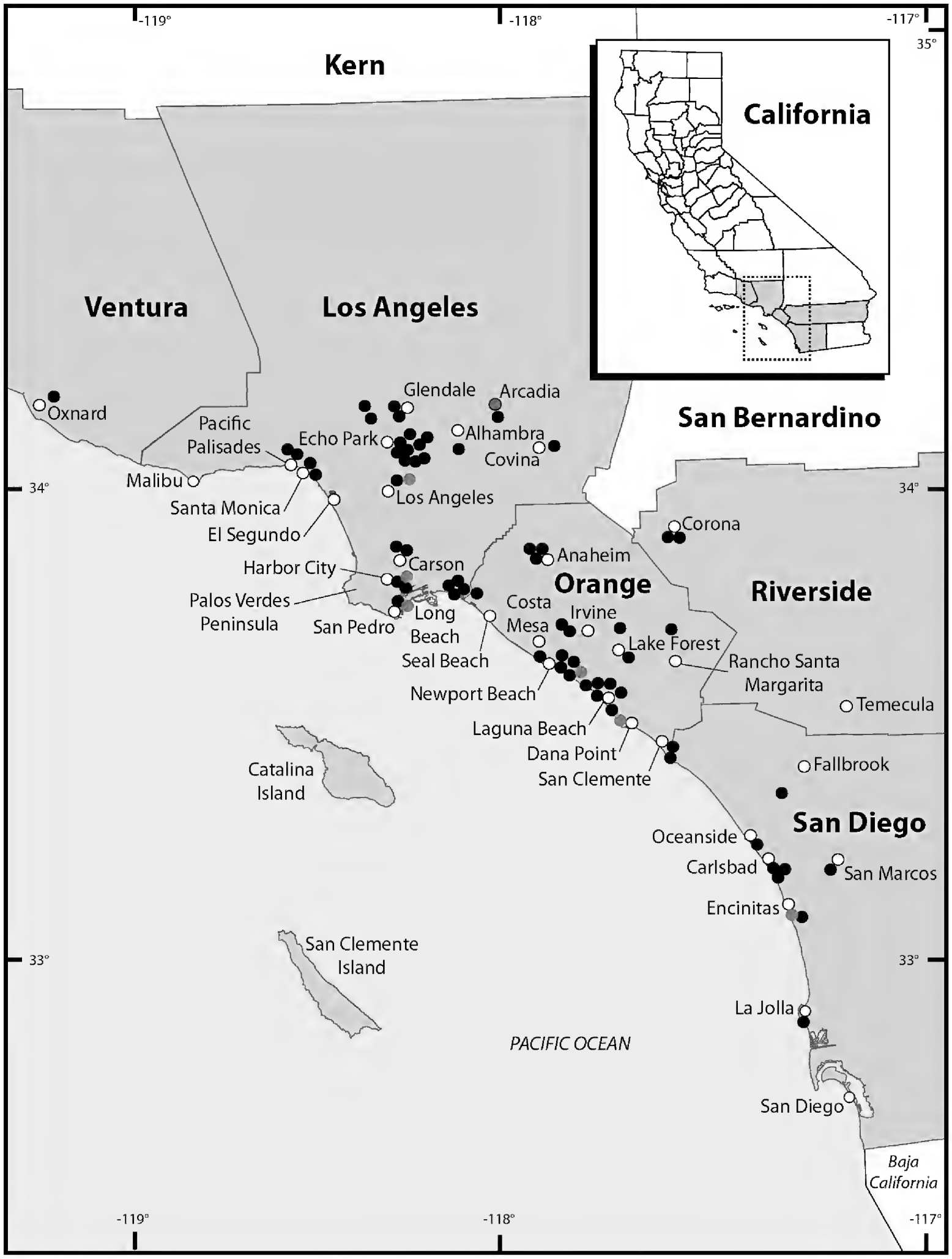

Figure 1. Known naturalized distribution of *F. microcarpa* in southern California: a solid circle (●) depicts locations documented in urban and native habitats; a red circle (●) identifies the largest of the naturalized trees observed during this study; and a blue circle (●) identifies the approximate location (Arcadia) of the first documented record of its pollinating fig wasp, *E. verticillata*, for California.

Figure 2. View of an approximately 5 m tall *F. microcarpa* tree growing on rough-grouted concrete wall at Harbor City, Los Angeles County. Note aerial and adventitious roots and tree base that has been cut back during landscape maintenance activities. Inset photograph showing mature syconium.

Figure 3. Approximately 3.5 m tall *F. microcarpa* tree growing from the side of a concrete reinforced bulkhead along harbor shores at San Pedro, Los Angeles County. Inset photograph showing mature and developing syconia. The Los Angeles Maritime Museum is in the background.

Figure 4. View of an approximately 3.2 m tall *F. microcarpa* tree rooted in an expansion joint of the 43rd Street bridge on the 110 Freeway, City of Los Angeles. Note water stains on bridge and retaining wall from urban runoff, and cultivated *F. microcarpa* tree planted along Flower St. the in upper right-hand side of photograph. Inset photograph showing immature syconia.

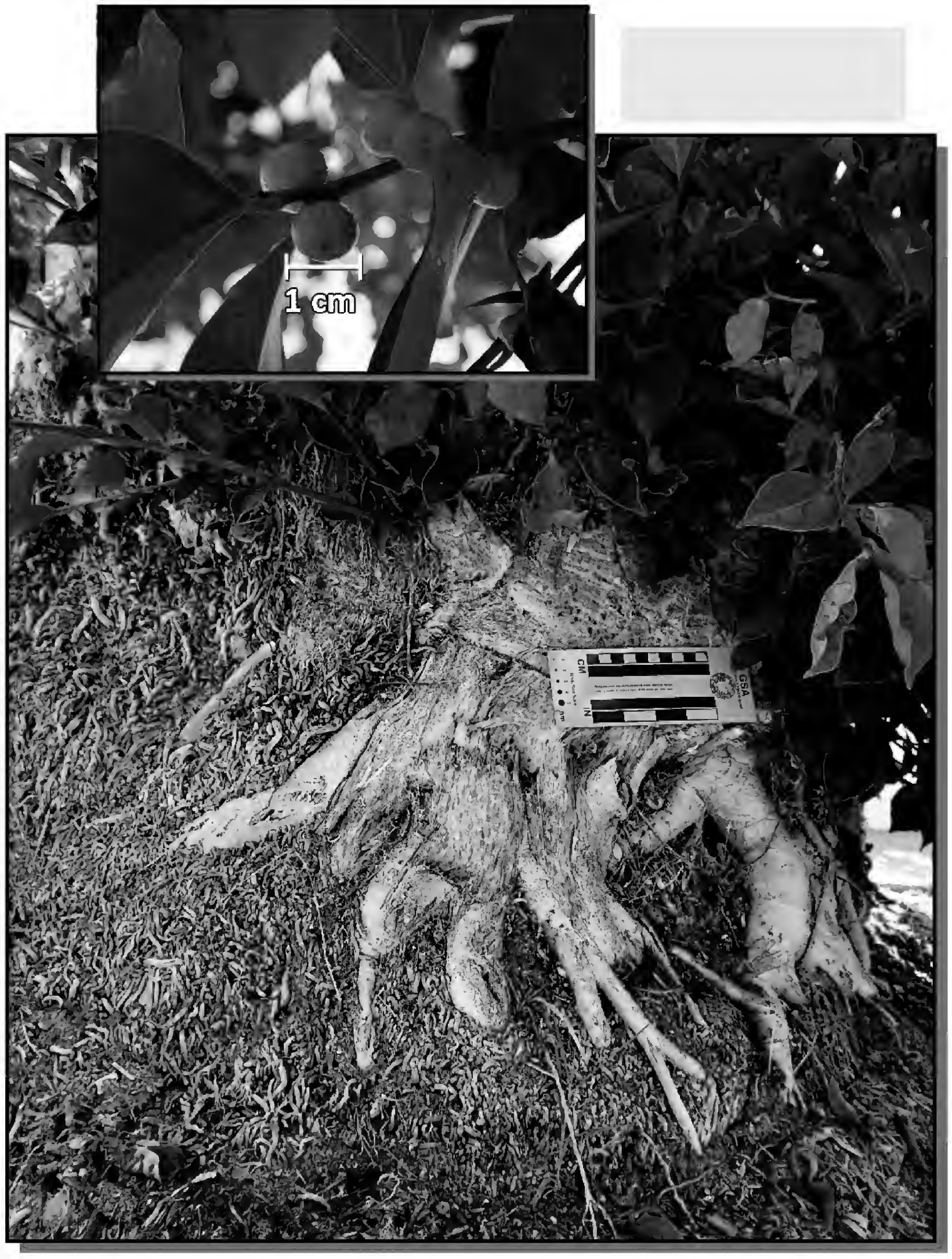

Figure 5. View of an approximately 3 m tall *F. microcarpa* tree growing on the base of a cultivated *P. canariensis* palm tree in Newport Beach, Orange County. Note the base (ca. 28 cm wide) of *F. microcarpa* has been cut back during landscape maintenance activities. Inset photograph showing immature syconia.

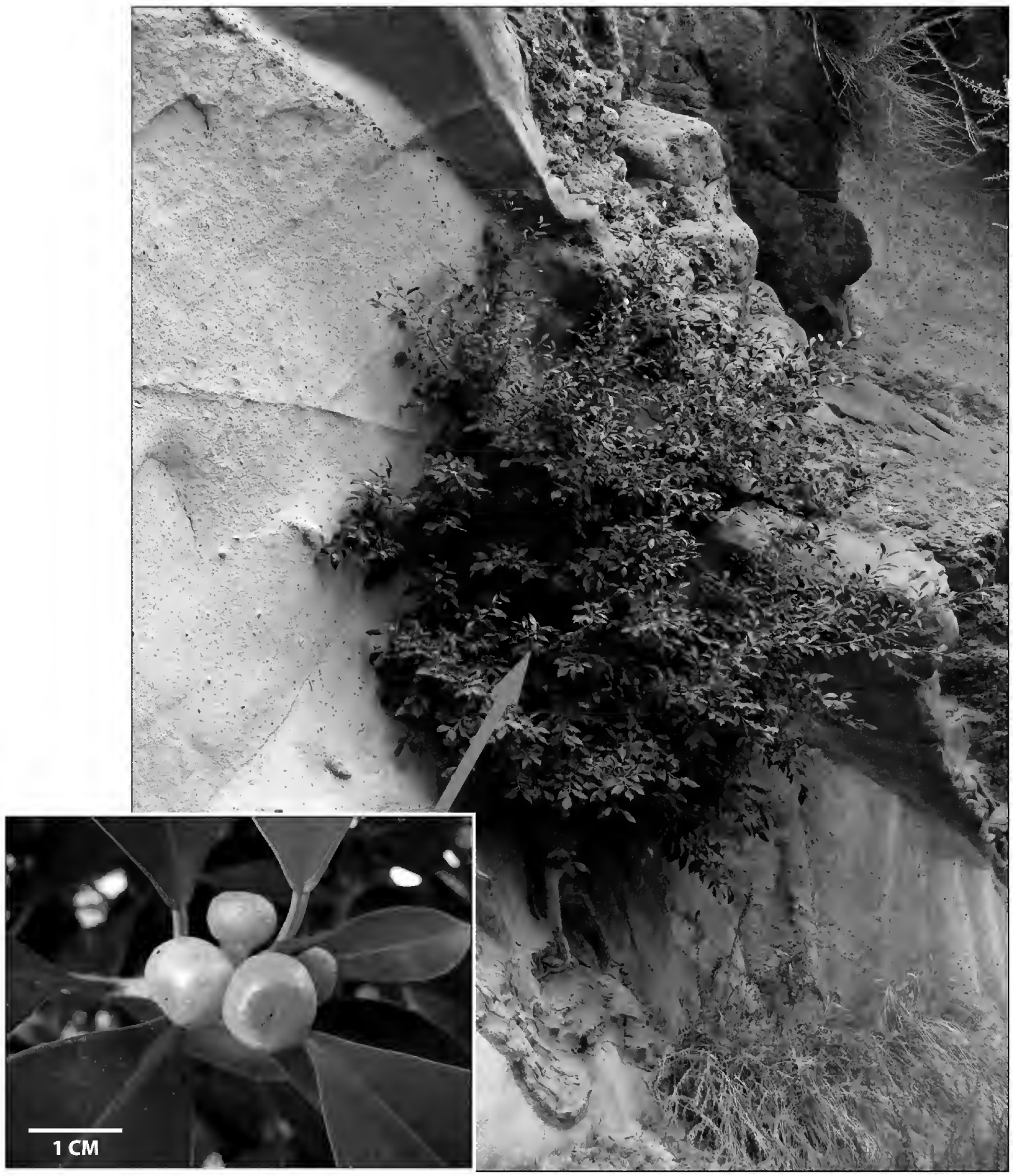

Figure 6. View of an approximately 3 m tall *F. microcarpa* tree growing at the base of a calcareous-saline sandstone cliff in Dana Point, Orange County. Many *Ficus* species that are hemi-epiphytes are also lithophytes, thereby enabling colonization of many urban and native habitats in southern California. Inset photograph shows immature syconia.

Figure 7. *Ficus microcarpa* growing on the trunk of *P. canariensis* cultivated in Encinitas, San Diego County. Note *F. rubiginosa* growing on the upper trunk.

Figure 8. View of *F. microcarpa* growing at the base of *Washingtonia robusta* in Oceanside, San Diego County. This was the smallest fertile plant observed during the study.

Figure 9. *Ficus microcarpa* grows in joints of old masonry and crumbling brick walls, Laguna Beach, Orange County. Note the base has been cut back during landscape maintenance activities.

Figure 10. View of *F. microcarpa* juvenile plant growing on the trunk of *P. canariensis*, Corona City Park, western Riverside County. Plants at the inland extremes of its current range may be influenced by aerosol drift from urban irrigation spray heads.

Figure 11. *Ficus microcarpa* grows in calcareous-saline soils on the bank of a tidal urban channel, Dominquez Channel, Carson, Los Angeles County. Perennial forbs in the photograph are *Parietaria judaica* L. (Urticaceae), another urban weed occasionally found in coastal native habitats.

Figure 12. *Ficus microcarpa* occurs rarely in native plant communities along the immediate coast. This approximately 1 m tall shrub grows on a calcareous-saline outcrop in Laguna Beach, Orange County. Note ephemeral seepage and salt crust formation on the outcrop surface.

Figure 13. *Ficus microcarpa* (*Riefner 15-135 photographic voucher*) grows on the upper trunks of palm trees, mostly *P. canariensis,* at inland and coastal localities. Moist coastal breezes, ocean fogs, and perhaps the inland extent of the marine layer, likely provide moisture to aid seedling establishment and persistent growth of *F. microcarpa* hemi-epiphytes growing in arid southern California. Inset photographs depict point of basal attachment and immature syconia. Photographs were taken in Carlsbad, San Diego County.

Figure 14. *Ficus microcarpa*, a strangler fig, is epiphytic on a palm tree trunk with *Nephrolepis cordifolia* in Long Beach, California. Note persistent leaf bases of *Butia capitata*. Inset photograph shows root basket formation typical of strangler figs that constrict and gradually kill host trees.

Why is *Verbesina virginica* (Frostweed, Asteraceae) not found in grasslands?

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ABSTRACT

Factors determining a species ecological niche are difficult to identify. *Verbesina virginica* (Frostweed, Asteraceae) is widespread across eastern North America but not generally found in grasslands or savannas. It usually occurs under a canopy or at the edge of a canopy. In central Texas it is found below the canopy of *Quercus virginiana* (live oak), *Ulmus crassifolia* (cedar elm) and a few other species. In order to better comprehend conditions limiting its distribution and niche requirements, a factorial experiment was performed. Three factors were examined including canopy (+ or -), additional water (+ or -) and neighbors (+ or -). Response variables were mortality, stem diameter, plant height, number of leaves, area of the largest leaf and aboveground dry mass. Total plant survival at the end of the experiment (234 days) was 28% or 27/96 plants. Survival was greatest below the canopy at 48% (23/48), while in the open it was 8% (4/48). Survival was greatest below the canopy in the no water, no neighbors treatment at 83%, but with no neighbors and additional water it was 50%. In the no canopy treatment, survival was 0% with the no water, no neighbors treatment and 11% with water, but no neighbors. Aboveground dry mass produced below the canopy was 64.7 g with a mean of 2.9±1.5 g/plant. Aboveground dry mass in the open was 9.1 g with a mean of 2.3±1.0 g/plant. Survival and dry mass was greatest for plants below the canopy, with no supplemental water and no neighbors. Survival was lowest when neighbors were present in the open or below the canopy and with or without supplemental watering. *Verbesina virginica* is mostly found in canopy shade because of the lack of C4 grasses and other herbaceous plants that probably take up water more efficiently during the hot-dry time of year. Thus, it is not found in canopy gaps because of the growth, competition and probably water uptake and use by the high temperature tolerant C4 grasses. Published on-line www.phytologia.org *Phytologia 98(1): 76-88 (Jan 5, 2016).* ISSN 030319430.

KEY WORDS: *Verbesina virginica* (Frostweed, Asteraceae), growth, competition, habitat preference, prairie, mortality, survival, niche requirements

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When a species is found in a given location, it is because that species can tolerate or requires the conditions present in that area. It is difficult to determine a species niche requirements, but more difficult to decide why it is not found in another place with similar conditions. Measuring density of terrestrial plants is relatively easy to do (Van Auken et al. 2005), but sorting out the factors that govern why a species is present where it is found is much more challenging (Begon et al. 2006). Some species are found in specific communities or at the edge of a community, whereas others are not constrained. Species may be limited spatially or temporally by abiotic or biotic factors or combinations (Begon et al. 2006; Leonard and Van Auken 2013; Louda and Rodman 1996; Maron and Crone 2006; Valladares and Niinemets 2008), but limiting factors may not be easily visualized.

Verbesina virginica seems restricted to growing below a canopy in shade (Gagliardi and Van Auken 2010). However, high carbon uptake in high light suggests *V. virginica* should be able to grow in high light, non-shaded open areas. When open grasslands, savannas or gaps were examined, *V. virginica* was not present. Similar situations have been reported for other species. For example, *Strepthanthus bracteatus*, a rare mustard found only in central Texas, occurred below a *Quercus verginiana/Juniperus ashei* (live oak, Fagaceae/ashe juniper, Cupressaceae) canopy unless the plant was protected from herbivory (Leonard and Van Auken 2013). Another mustard (*Cardamine cordifolia*, bittercress) was restricted to shaded habitats because of chronic insect herbivory in full sun (Louda and Rodman 1996). Many studies have indicated herbivory can have major effects on plant abundance, dynamics, distribution and community composition (see Maron and Crone 2006).Woody plants have long been restricted from most grasslands because of high fire frequency (see Collins and Wallace 1990; Van Auken 2000), but with the introduction of large numbers of domestic herbivores, fuel mass has decreased as has fire frequency, with a concomitant increase of woody plants in grasslands (Van Auken 2000, 2009).
Unfortunately, the reason that *V. virginica* is not present in grasslands is undefined.

In central Texas, savannas are associated with grasslands, woodlands or forests (Van Auken and McKinley 2008; Van Auken and Smeins 2008). A species found in some of these woodland communities or edge communities is *V. virginica* L. (Frostweed, Asteraceae) (Correll and Johnston 1979; Strother 2006). It appears to be an understory species, sometimes forming almost mono-specific communities (Gagliardi and Van Auken 2010). It can establish below some species, but no studies were identified concerning which species, its light requirements, essentials for establishment or successional status (Enquist 1987).

Light level is an important factor limiting or controlling the presence of many species in various communities (Begon et al. 2006; Smith and Smith 2012). Species growing in shady habitats have reduced photosynthetic rates, lower light saturation, light compensation points and dark respiration compared to those growing in full sun (Boardman 1977; Begon et al. 2006; Larcher 2003; Valladares and Niinemets 2008). Adaptive crossover is displayed by some species allowing them to acclimate to high or low light environments and have a broader ecological niche (Givnish et al. 2004).

Verbesina virginica had a fairly high photosynthetic rate which was surprising for a species found in shade below a canopy (Gagliardi and Van Auken 2010). A related species, *V. encelioides*, had an *Amax* of 12.3 μmol $CO_2/m^2/s$, which is within the range reported for *V. virginica*, but *V. encelioides* is a disturbance species and not expected to do well at low light levels below a canopy (Gleason et al. 2007). Another species, *V. arborea*, a tropical species, grew well in open grazed plots where seed was added, suggesting it was a sun species as well (Posada et al. 2000). Our conundrum was why did *V. virginica* have an unusually high photosynthetic rate and grow in low light below a canopy, but not in adjacent grasslands?

PURPOSE

Our hypothesis was that *V. virginica* did not compete well with associated herbaceous species and consequently was forced into a secondary habitat where it survived and grew quite well because the C4 grasses were not present and the shade adapted C3 species were limited.

METHODS

This study was carried out in the City owned Phil Hardberger Park in San Antonio, Texas, USA

(N-29°33"41.3", W-98°31'11.8"). Most of the subsurface of the area is Cretaceous limestone, and soils are usually shallow, rocky or gravelly, dark colored, calcareous with neutral or slightly basic pH, usually Austin silty clays, Whitewright-Austin complex, or Eckrant cobbly clay (Taylor et al. 1962; NRCS 2006).

The area is approximately 20 km south of the Edwards Plateau region of central Texas just south of the Balcones Escarpment in northern Bexar County (Correll and Johnston 1979; Van Auken et al. 1981; Van Auken and McKinley 2008). The elevation of the study area is approximately 350 m above mean sea level (AMSL) (Taylor et al. 1962; NRCS 2006). Mean annual temperature is approximately 20.0°C with monthly means from 9.6°C in January to 29.4°C in July (NOAA 2004). Precipitation is 78.7 cm/yr, bimodal, with peaks in May and September (10.7 cm and 8.7 cm), little summer rainfall, high evaporation and high variability (Thornthwaite 1931; NOAA 2004).

Verbesina virginica L. (Frostweed, Asteraceae) can be up to 1.8 m tall, and is an erect, unbranched, herbaceous, perennial plant with the main stem prominently winged (Figure 1A and B). It is frequently found in the eastern United States and its western limit of distribution is Kansas, Oklahoma and Texas (Correll and Johnston 1979; USDA 2009). In central Texas, it is mostly found beneath the canopy of *Quercus virginiana* (live oak, Figure 1C), Q. *stellata* (post oak), Q. buckleyi (Texas red oak), *Ulmus crassifolia* (cedar elm) and *Juniperus ashei* (ash juniper), usually on deeper soils in some of these communities (Gagliardi and Van Auken 2010). Its common name comes from ice crystals that surround the stem usually after the first freeze (Figure 1D).

Verbesina virginica can form mono-specific communities in understory habitats especially on deeper soils including some riparian soils. Isolated plants are occasionally found below the canopy in some upland central Texas communities (Enquist 1987). Leaves are large and ovate to oblong-lanceolate and pubescent. Flowering is in late summer concluding with cold temperatures and frosts in late fall. The flower heads usually have three to four white to greenish white ray flowers and up to 15 disk flowers. It tolerates high temperatures but leaves are usually wilted during dry conditions. The rooting system is unreported but is probably a deep tap root and we do not think the plants are connected via rhizomes.

Area vegetation in this region was savanna or woodland with *Juniperus-Quercus* (juniper and oak) communities being dominant, but higher in woody plant density than communities farther to the west (Smeins and Merrill 1988; Van Auken et al. 1981; Van Auken and McKinley 2008.). High density woody species are *Juniperus ashei* (Ashe juniper) and *Quercus virginiana* (=*Q. fusiformis*, Live oak) followed by *Diospyros texana* (Texas persimmon) and *Sophora secundiflora* (Texas mountain laurel). *Ulmus crassifolia* (cedar elm) is found in these communities, but usually at lower density and on the deeper soils. There are also former grasslands of various sizes that are woodlands today with *Prosopis glandulosa* (mesquite), *Aloysia gratissima* (whitebrush) and *Diospyros texana* as major woody species. These areas seem to be on deeper soils and were not used in the current study. Within the *Juniperus- Quercus* woodlands there are sparsely vegetated intercanopy patches or gaps on shallow soil (openings in the woodlands) (Van Auken 2000). This is where the high light or open treatments were placed.

The most important herbaceous species below the canopy are *Carex planostachys* (Cedar sedge) (Wayne and Van Auken 2008) and *V. virginica* (Gagliardi and Van Auken 2010). In the gaps, *Aristida longiseta* (Red three-awn), *Bouteloua curtipendula* (Side-oats grama), *Bothriochloa* (=*Andropogon*) *laguroides* (Silver bluestem), *B. ischaemum* (KR bluestem), various other C_4 grasses, and a variety of herbaceous annuals are common (Van Auken 2000).

Experimentally, a three factor, factorial experiment was set up. The factors were canopy or no canopy (+ or - canopy), added water or no added water (+ or - water), and neighbors or no neighbors (+ or - neighbors). There were two physical locations, two levels of added water and neighbors were present or

removed. The experiment included 12 replications for each treatment.

Plants were started from seed and grown for 60 days in 10.1 x 10.1 cm peat pots (in a greenhouse) in native area soil from the study site (dried, sifted Whitewright-Austin complex) with 100 ml of a complete nutrient solution added initially (Van Auken, et al. 2005). There were 12 replications of each treatment for a total of 96 pots or plants (2 positions, 2 water treatments, 2 neighbor treatments, and 12 replications or 2x2x2x12=96 total pots or plants). Plants were randomized and planted in the field March 7, 2013. All plants were watered initially and then every other day with 500 ml of tap water for two weeks. After that, only the water + treatment plants were given tap water and only once/week.
Watering was done to maintain the soil at approximately field capacity. Basal diameter, height and number of leaves as well as the size of the largest leaf was measured monthly. Stem and leaf area were calculated. Live and dead plants were counted monthly. Upon harvesting, when growth had stopped (day 234), shoots were clipped at the soil surface and dried at 75^{o}C to a constant level and then mass was determined. Roots were not collected. Light levels were measured at each plant position using a LI-COR® LI-190 SA integrating quantum sensor. A total of 96 measurements were made, and values were averaged for each position (Van Auken 2000).

Analysis of variance was used for final results (Sall et al. 2001). This was used to test the effect of canopy position, added water and the presence of neighbors on response variables. Interactions that were not significant were removed from the models. Least square regressions were completed to examine how mortality and other response variables changed in time. Data were compared to various functions.
Significance level for all tests was 0.05.

RESULTS

The experiment was planted on March 7, 2013 and harvested 234 d later on November 1, 2013. Overall mortality, at the end of the experiment, was 72% or 69/96 dead and survival was 28% with 27/96 total plants surviving. Mortality of *Verbesina virginica* increased through the experiment (Figure 2) and was greatest in the open or full-sun at 92% (44/48) with four survivors. Below the canopy in shade, mortality was 52% (25/48) with 23 of 48 plants surviving or 48% survival. Mortality was a significant linear function (Figure 2) and transformations did not significantly increase the coefficient of determination (R^2) or P value (not presented). Time (days) explained 90-95% of the variation of total and below canopy mortality of *V. virginica* (*Figure 2*).

Four plant growth factors were measured during the experiment including plant height, number of leaves, length and width of the largest leaf and basal stem diameter. Largest leaf area was calculated as was stem basal area. These factors were regressed on time in days that they were measured or counted.
Linear as well as logarithmic and polynomial (2^{nd}, 3^{rd} and 4^{th} order) regressions were examined. None of the linear and logarithmic regressions were significant (P>0.05 in all cases).

Height for all living plants was significant as a 2^{nd} degree polynomial (Figure 3). The R^2 for all plants was 0.58. For plants growing below the canopy it was 0.66 and for plants grown in the open (no canopy) it was 0.47. Thus, the R^2 for a 2^{nd} degree polynomial function explained 47-66% of the variation in height of *V. virginica* over the time in days of the experiment (Figure 3).

The number of leaves, leaf area and mean basal stem diameter were all significantly related to time but were 2^{nd}, 3^{rd} or 4^{th} order polynomial functions. These polynomial regressions explained 57-86% of the variation of that factor in time. All measured factors increased from the start of the experiment in March of 2013 through the spring months and reached a peak in late June (on day 107) and then

declined through late summer and fall until the experiment was terminated and plants were harvested in November (on day 234). *Verbesina virginica* plants were largest on the 107th day of the experiment (Table 1), with plants in the open (no canopy) being the tallest, had the greatest number of leaves and stem basal diameter, but leaf area of the largest leaf was the same for plants from both positions, but differences were not significant.

Table 1. Mean height, number of leaves, area of the largest leaf and stem basal diameter of *Verbesina virginica* plants on June 27, 2013 the 107th day of the experiment when plants were largest.

	POSITION	
RESPONSE VARIABLE	CANOPY	NO CANOPY
HEIGHT (cm.)	12.7	15.5
NUMBER OF LEAVES	8.0	10.3
AREA-LARGEST LEAF (cm^2)	44.2	44.3
STEM BASAL DIAMETER (mm.)	2.66	3.25

Considering final plant dry mass, canopy position was a significant main effect in the experiment, while added water or the removal of neighbors were not significant (Table 2). The total number of live plants at the end of the experiment below the canopy and in the open (no canopy) are shown in Figure 4A. The largest number of live plants was below the canopy and that is where the greatest dry mass was produced (Figure 4B). However, there was a significant position (+ or - canopy) x neighbor (+ or -) interaction (Table 2, Figure 5A). If plants were in the open (- canopy), with or without neighbors, dry mass was less than one gram per plant. If plants were below the canopy with neighbors, dry mass was about 2 grams per plant, but with neighbors removed, dry mass was 3.45 g/plant. Survival of *V. virginica* plants with neighbors and no canopy was three plants (Figure 5B), but was one plant if neighbors were removed. Below the canopy with neighbors, survival was nine plants, but if neighbors were removed, survival was 13 plants.

In the bar graph showing all of the treatments, dry mass was greatest in the two canopy treatments without neighbors (Figure 6A). Dry mass was highest in the +canopy treatments and lowest in the – canopy treatments. The number of live plants was greatest in the + canopy treatment and lowest in the – canopy treatments (Figure 6B).

Table 2. ANOVA table with results comparing *Verbesina virginica* plant dry mass with canopy position (canopy no canopy), water (added water or none added), neighbors (present or removed) and their interactions are included. *F*-ratio and *P* values are presented in the table with significant *P*-values in bold and *.

Source	*F*-Ratio	*P*-value
Canopy (C)	19.2710	**<0.0001***
Water (W)	0.0091	0.9224
Neighbors (N)	2.0386	0.1568
C x W	0.2322	0.6311
C x N	4.9024	**0.0294***
W x N	1.2795	0.2611
C x W x N	1.2297	0.2705

When the sum of the final aboveground dry mass was examined, there was seven times as much dry mass below the canopy (64.7 g + canopy or in the shade) compared to the – canopy or open, full sun treatment with only 9.1 g. The mean above-ground plant dry mass below the canopy was 2.9±1.5 g/plant and mean above-ground plant dry mass in the open was 2.3±1.0 g/plant. Dry mass was greatest for plants below the canopy, with no neighbors (Figure 6A and B). Survival was lowest when neighbors were present in the open or below the canopy and without supplemental watering. The sum of the dry mass when neighbors were removed was twice as high below the canopy compared to when neighbors were not removed (Figure 7).

Verbesina virginica survival was greatest in canopy shade where soil was approximately 50% deeper (Table 3) and light levels were 5.7% of light levels in the open position (–canopy).

Table 3. Comparison of light levels and soil depth for *Verbesina virginica* with an *F*-test with canopy position (canopy no canopy) as the main treatment. Means, standard deviations, *P*-values and percent of no-canopy values are presented in the table with significant *P*-values in bold and *.

	CANOPY	%	NO CANOPY	*P*-VALUE
LIGHT LEVEL(μmoles/m^2/s	108±125	5.7	1905±303	**<0.0001***
SOIL DEPTH (cm.)	13.48±6.80	150.7	8.94±4.49	**<0.0053***

DISCUSSION

During this and previous studies, *Verbesina virginica* was found below or at the edge of the canopy of *Q. virginiana* or *U. crassifolia* (Figure 1), but not in associated grasslands. Planting *V. virginica* in the open (no canopy) resulted in high mortality (Figure 2). No *V. virginica* plants were seen in the grassland during this study or in a previous study (Gagliardi and Van Auken 2009), but reports from the literature are not consistent concerning where it is found (Enquist 1987; Strother 2006; USDA 2009).

Light levels appeared to be important, with almost no *V. virginica* plants found in the high light open grassland habitat and few survived if they were planted there (Figure 4). Additionally, grassland soil was not as deep as the soil below the canopy (Table 3). Furthermore, *V. virginica* was not expected in the more shallow soils of the arid upland communities (Van Auken et al. 1981).

The presence of neighbors was also important and possibly the most important factor in determining the presence of *V. virginica*, but it was not a significant main effect in the current experiment (Table 2). Thus, the presence of neighbors seem to be a more subtle but not less important factor in influencing or determining the presence, density and the distribution of *V. virginica* in these communities. Neighbor effects seemed to be combined with one or more other factors, thus an interaction. The various C4 grasses in the open and the C3 sedge, *Carex planostachys* below the canopy may be more efficient in taking up water and possibly nutrients and thus reduce the possibility of *V. virginica* easily establishing in these habitats (Wayne and Van Auken 2009). The inhibiting effects of the C4 grasses seems to be paramount, but may be transitory and the high mortality of all *V. virginica* plants in the grassland habitat prevented us from teasing apart the potential neighbor and water effects in the current experiment (Figure 4). We don't know how long understory *V. virginica* plants would persist if the canopy were removed.

Finding positive and negative interactions between species is not unusual (Harper 1977; Grace and Tilman 1990, Fargione and Tilman 2005, Elliott and Van Auken 2014), but demonstrating the potential cause of the effect is much more difficult to do (Louda and Rodman 1990; Begon et al. 2006; Marion and Crone 2006; Valladares and Niinemets 2008; Smith and Smith 2012; Leonard and Van Auken 2013). Usually multiple abiotic factors interact to control the kinds of plants present in a given habitat.

However, it is the species response to these abiotic factors and their biotic interactions with them that will determine the community composition. These factors are dynamic and individuals are responding to them all of the time which makes it difficult to know which one or ones are controlling their responses and thus community composition. Because a species is present in a community does not mean it was there yesterday or will be there tomorrow.

Light levels and a species response to them are easy to understand singly, but when a species responds to other factors and other species at the same time, understanding or disentangling which factors are most important, if any, is difficult. Shade leaves of *V. virginica* plants in the low light environments of canopy trees, were capable of a high maximum photosynthetic rates (A_{max}), which is not typical of species growing below a canopy (Begon et al. 2006). Shade adapted leaves of various eastern deciduous forest understory species usually had A_{max} values lower than those reported for *V. virginica* (Hull 2002; Gagliardi and Van Auken 2010). Other photosynthetic parameters reported for *V. virginica* were in the range expected for shade adapted plants not sun species (Valladares and Niinemets 2008), but they were measurements of shade, not sun leaves.

Verbesina virginica has a fairly broad distribution, especially in the eastern United States. But very little is reported about its growth responses to light levels or other environmental factors. Most of the parameters measured for shade leaves suggest that this species is a shade adapted species, but A_{max} rates do not agree suggesting it can grow in full sun where we didn't find it and almost all of the plants placed or grown in full sun or open habitats died. Usually, true understory species have much lower photosynthetic rates than the rates previously reported for *V. virginica*. For example, *Carex planostachys* from the central Texas Edwards Plateau *Juniperus* woodland understory had an A_{max} value of 4.9 ± 0.3 $\mu molCO_2/m^2/s$ which was lower than the A_{max} for shade leaves of *V. virginica* and reached light saturation at low light levels (Wayne and Van Auken 2009). While *V. virginica* in central Texas is typically found growing in shaded habitats or the edge of woodlands, its high A_{max} for shade adapted leaves compared to other herbaceous shade plants would suggest it could grow in a variety of light environments including open habitats, but it was not found there.

Some plants can occur in a variety of light environments including some plants from disturbed (open) communities growing in shade (Bazzaz and Carlson 1982). Plants like *V. virginica* that have a relatively high A_{max} that changes little over a wide range of light levels could do well in shade with the presence of sunflecks (Hull 2002). However, there is nothing in the literature about *V. virginica* and its ability to grow in variable light. Stomatal conductance and transpiration reported for *V. virginica* previously were similar to a number of other species, but not compared with the native C4 grasses (Gagliardi and Van Auken 2010). Xylem water potential of this species has not been measured or compared. Water use efficiency of this species should be examined closely with and without C4 neighbors and at high and low light levels (Larcher 2003; Grunstra and Van Auken 2015). Results of studies like this would help determine why *V. virginica* is not found in open grasslands.

Verbesina virginica showed interesting photosynthetic responses in previous studies (Gagliardi and Van Auken 2010). These physiological responses to various light levels more than likely are contributors to the apparent niche observed for this species in the field. In general, resource utilization is spatially partitioned among species along environmental gradients, such as changes in light from open areas to woodland or forest edges (Wayne and Van Auken 2009; Gagliardi and Van Auken 2010). The ability of *V. virginica* to reach high photosynthetic rates at lower light level, its light saturation, and light compensation point allow it to exist in shaded environments. At light levels below 300 $\mu mol/m^2/s$, data suggests that other more shade tolerant species such as *C. planostachys* would probably be able to out-compete *V. virginica* (Wayne and Van Auken 2009), but not after *V. virginica* was established because

of the deep shade below its canopy. At light levels above 300 µmol/m^2/s below the canopy, *V. virginica* could dominate, in part because it has photosynthetic rates as high as or higher than most co-occurring species and its large leaves would reduce light levels to very low values below its canopy (Grunstra 2008; Furuya and Van Auken 2009; Wayne and Van Auken 2009). However, its absence in associated grasslands is not explained. The established C4 grasses would have equal or higher photosynthetic rates, have higher water use efficiency and perhaps be more tolerant of higher light levels and lower soil water levels than *V. virginica*.

CONCLUSIONS

Frostweed survival was low in open areas without a canopy and highest in canopy shade. Below the canopy, removal of neighbors is important and suggests it is not a good competitor. It can establish and grow in full sun or open areas but seems to require a disturbance to do so. In addition there would have to be seeds present in the soil in order for it to take advantage of the disturbance, especially if the disturbance was small.

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Figure 1. Photographs of some *Verbesina virginica* plant characteristics. Floral characteristics are shown in (A) and flower heads have both disk and ray flowers. The wings that are present on the stem are shown in (B). A habitat photograph (C) shows *V. virginica* below the canopy of several live oak trees (*Quercus verginia*). The characteristic ice around the stem of *V. virginica* after a frost or freezing temperature is also shown (D). Photos were taken by J. Gagliardia.

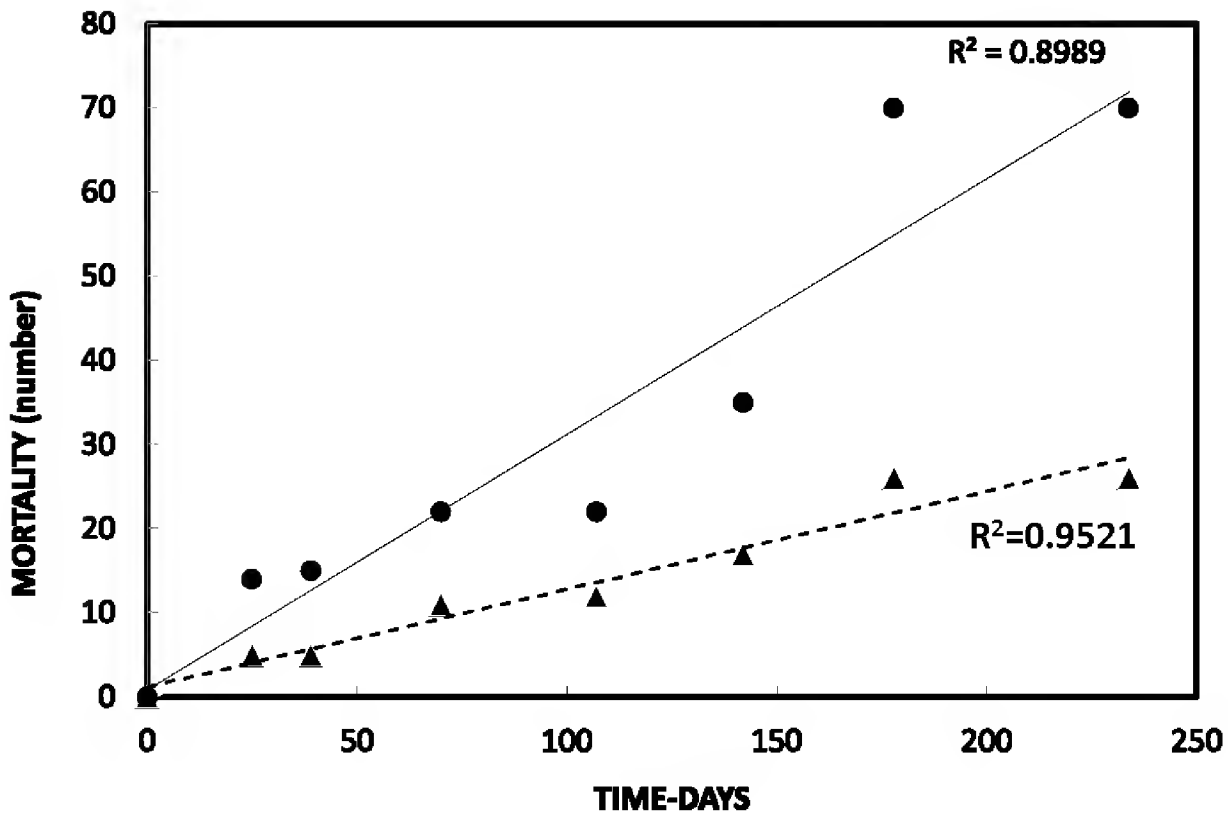


Figure 2. Mortality of *Verbesina virginica* plants at Hardberger City Park in San Antonio, Texas, USA (N-29°33"41.3", W-98°31'11.8"). Mortality of 96 plants (48 plants below a canopy and 48 plants in the open) were followed for 234 days in 2013. Total mortality is displayed (●) solid line and is a linear function (y=0.3036x + 0.8313, $P < 0.001$) as is mortality below the canopy (▲) with a dashed line (y=0.1164x + 1.1867, $P < 0.001$). Mortality was greatest in the open and increased to 92% or 44 out of 48 plants at the end of the experiment and is a linear function (y=0.1881x + 0.3223, $P < 0.01$) but is not shown.

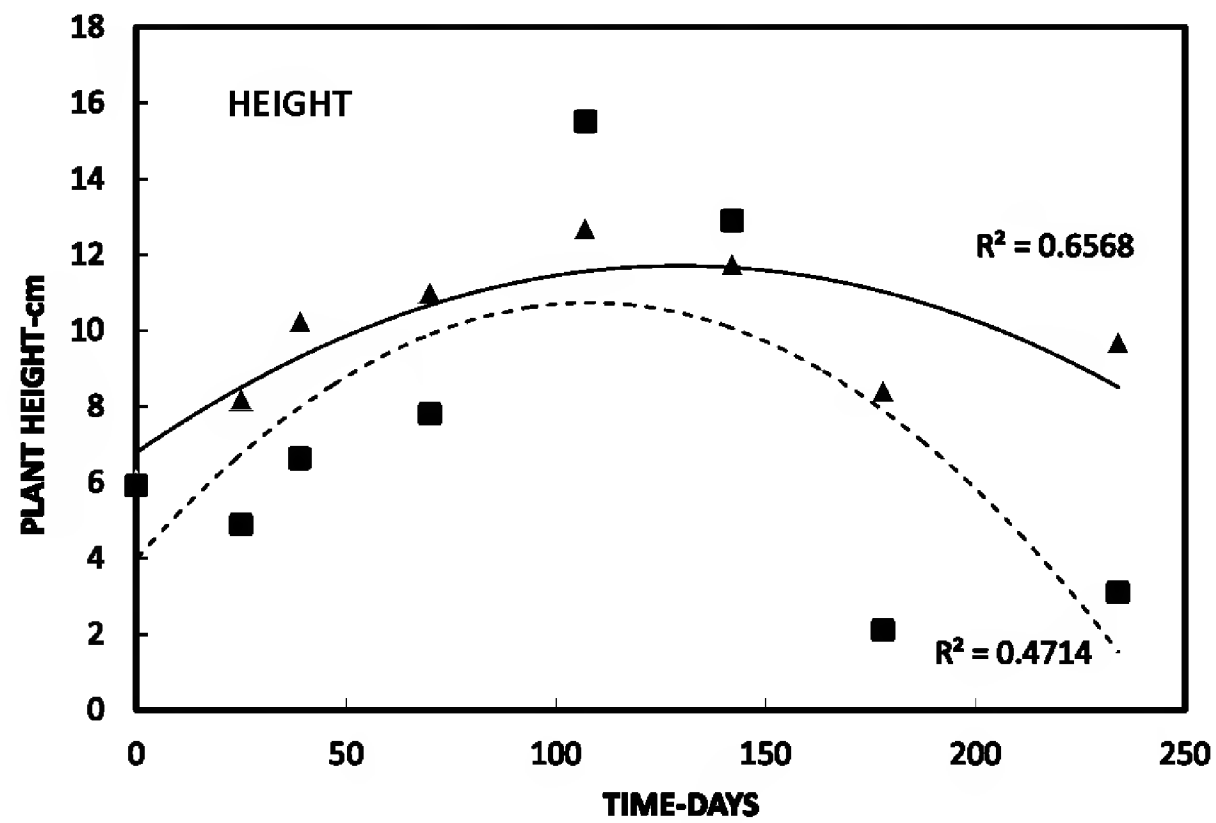


Figure 3. Height of *Verbesina virginica* plants at Hardberger City Park in San Antonio, Texas, USA. Height of plants was measured in centimeters approximately once per month over the course of the experiment. Lines are 2nd order polynomial functions and coefficients of determination (R^2) are presented. The triangles (▲) are for the plants below the canopy ($y = -\ 0.0006x^2 + 0.1251x + 3.97$, $P < 0.05$) and the squares (▄) and dashed line are for open grown plants (no canopy, $y = -0.0003x^2 + 0.076x + 6.78$, $P < 0.05$).

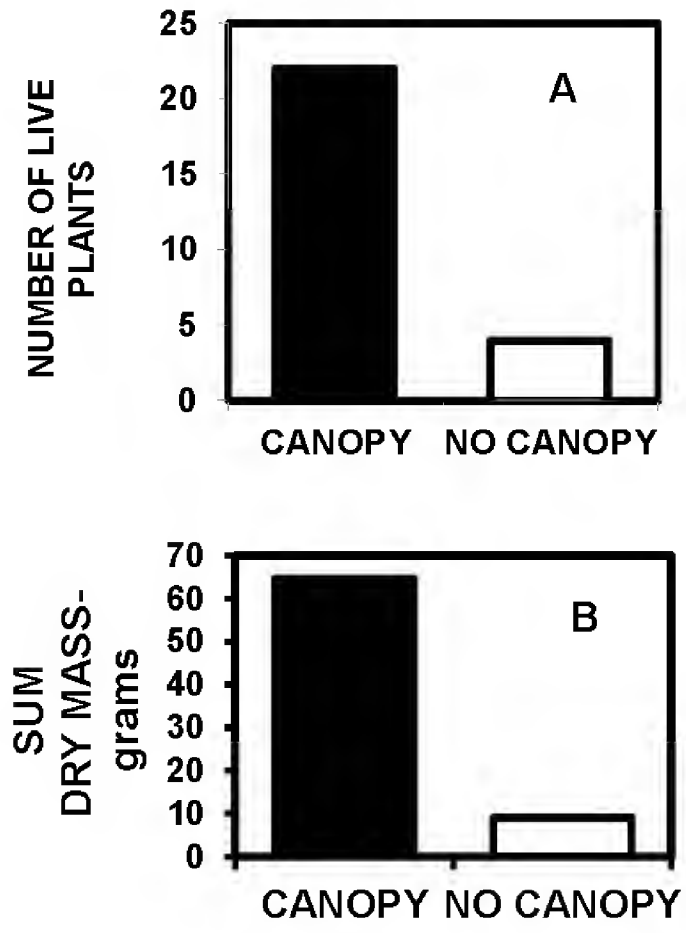


Figure 4. Number of live plants (A) at the end of the experiment below the canopy and in the open (No Canopy). Sum of live plant dry mass in grams (B) at the end of the experiment below the canopy and in the open (No Canopy)

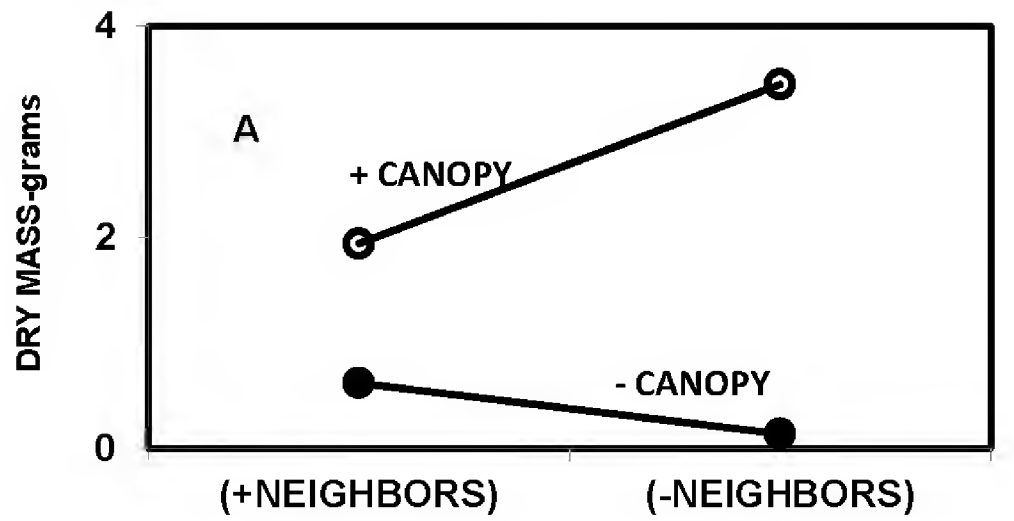
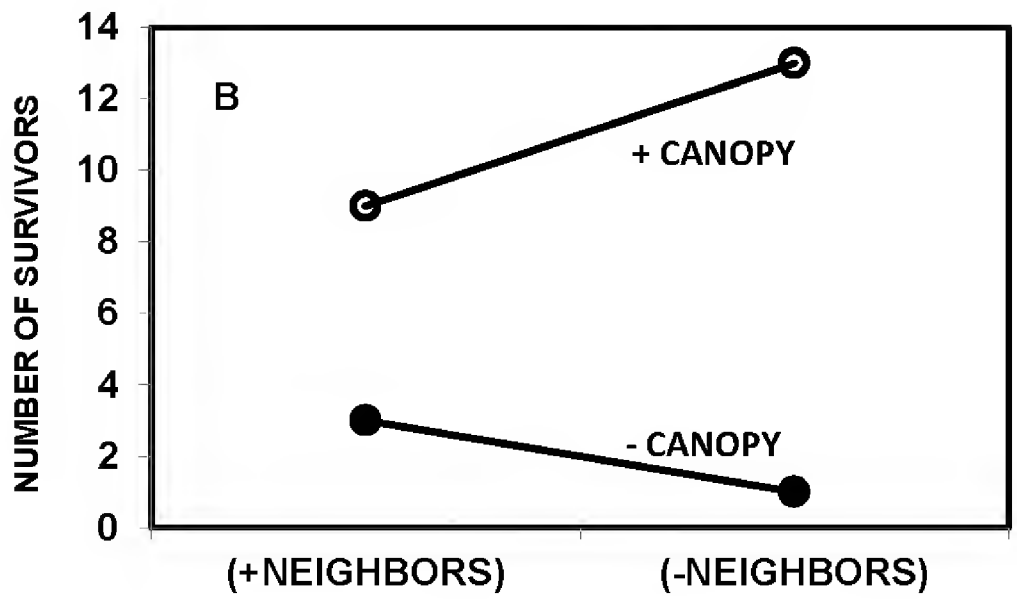
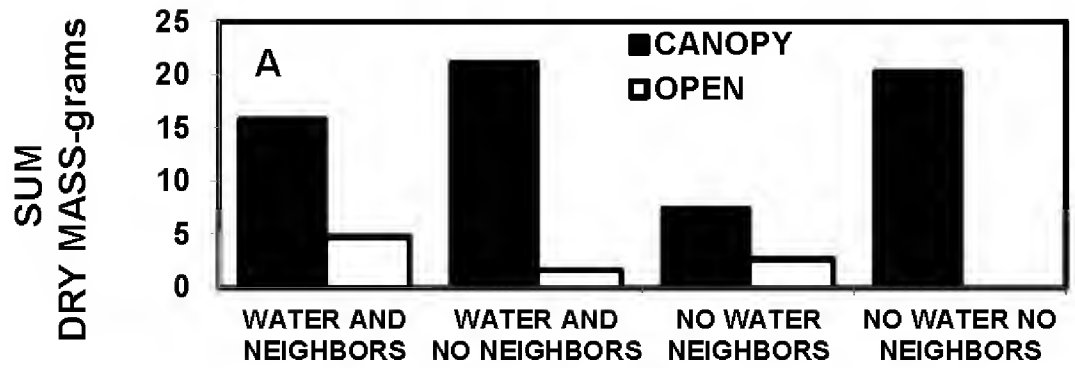


Figure 5. Interaction plot (A) of *Verbesina virginica* plant dry mass in grams with position +canopy or – canopy and + or – neighbors and (B) the number of survivors. Significant two way ANOVA interaction for (A) position and neighbors with F = 4.9024 and P = 0.0294 but (B) was not significant.





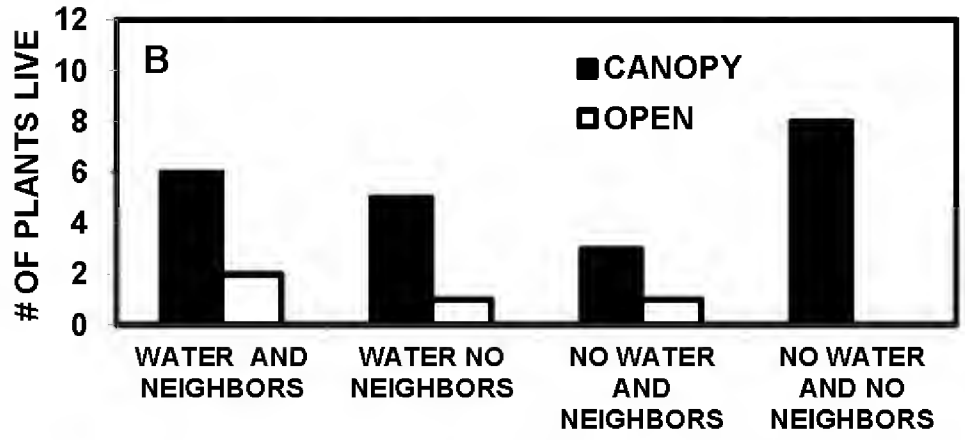


Figure 6. Sum of dry mass in grams (A) was greatest in the canopy treatment (black bars). Greatest dry mass was in the canopy and no neighbor's treatment with or with no added water. Total survival (B) was greatest in the canopy treatment (black bars) at 48% or 23/48. Greatest survival was in the canopy no water and no neighbor's treatment at 75% (9/12) and there were no survivors in the open in this treatment.

Figure 7. Sum of dry mass in grams produced by + canopy grown *Verbesina virginica* with no neighbors or with neighbors.